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B-cell acute lymphoblastic leukemia promotes an immune suppressive microenvironment that can be overcome by IL-12

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B-cell acute lymphoblastic leukemia promotes an immune suppressive microenvironment that can be overcome by Interleukin-12

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Abstract

B-cell acute lymphoblastic leukemia promotes an immune suppressive microenvironment that can be overcome by Interleukin-12

By Alisha Desiree “Rae” Hunter

Immunotherapies are a breakthrough in treatment for B-cell acute lymphoblastic leukemia (B-ALL), particularly in patients with relapsed or refractory disease. Thus, for my thesis project, we sought to identify mechanisms of immune suppression in high-risk B-ALL and strategies to overcome them. We first began by examining T- and myeloid cell responses when exposed to leukemia cell supernatant. We observed when T-cells were stimulated in ALL supernatant *ex vivo* there is a reduction in the surface expression of the T-cell activation marker, CD44, and CD107b, a degranulation marker. Macrophages co-cultured with leukemia cells were also unable to significantly upregulate activation markers, CD80 and CD86, compared to macrophages in unconditioned media.

Single-cell RNA-sequencing analysis of samples collected from patients with B-ALL with measurable residual disease (MRD) after induction chemotherapy revealed T-cell exhaustion. To investigate T cell exhaustion *in vivo* we used a mouse model of B-ALL and we observed reductions of T cell and dendritic cell numbers and activation similar to what is observed in MRD positive patients. Impressively, recombinant interleukin-12 (rIL-12) treatment of mice with B-ALL significantly increased the number of splenic and bone marrow resident T-cells and DCs. We also observed a shift to an immunostimulatory cytokine and chemokine bone marrow microenvironment in mice with B-ALL treated with rIL-12. Targeted RNA-sequencing of T-cells isolated from vehicle and rIL-12 treated mice with B-ALL provided mechanistic insight into how IL-12 treatment overcomes B-ALL induced immunosuppression. Genes associated with immune exhaustion, including *Lag3* and *Tigit*, were suppressed with rIL-12 treatment, relative to levels observed in vehicle-treated mice. In addition to the beneficial effects of rIL-12 treatment in mice with B-ALL, the cytolytic capacity of the immunotherapy blinatumomab, a bispecific engager, was also enhanced in co-culture experiments with human T-cells and B-ALL cells. In the presence of B-ALL secretome, blinatumomab efficacy is reduced and this suppression can be overcome with IL-12 treatment.

Overall, this work provides mechanistic insight into how IL-12 overcomes B-ALL-mediated immune suppression. This suggests the potential for novel treatment strategies utilizing IL-12 for the treatment of B-ALL.

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Chapter 1: Introduction

1.1 Cancer

1.1.1 Cancer Overview

Cancer is characterized by uncontrolled cell growth, invasion, and metastasis. Often, this is due to mutations that cause activation of genes, called oncogenes, or inactivation of tumor suppressor genes. This results in continued cell cycle progression with no “brakes” and apoptotic mechanisms no longer activated. Cancer development is dependent upon numerous changes in the tumor microenvironment and heterogeneous interactions between cancer cells and surrounding normal cells. Most types of cancers, if left unchecked, will undergo metastatic growth. Therefore, studying various tumor types and their dynamic relationship with the microenvironment is critical to treat patients. Unfortunately, cancer is still affecting populations worldwide. In 2022, it is estimated that 1.9 million new cancer cases will occur with 609, 360 patients predicted to succumb to cancer in the United States [1]. A better understanding of factors that promote cancer pathogenesis is critical to designing better therapies to treat this disease.

1.1.2 Hallmarks of Cancer

Essential alterations in cell makeup have previously been defined to understand the molecular processes that lead to cancer development. These alterations consist of cellular function changes including evasion of apoptosis, sustained angiogenesis, uninhibited growth, and tissue evasion and metastasis [2]. The majority of these capabilities are common among various types of cancer. In 2011, emerging and enabling hallmarks were described to recognize the significance of the immune system in addition to describe metabolic alterations that occur in cancer cells [2]. As a result, cancer immunotherapy has now been a significant area of pursuit. Although the hallmarks describe shared traits among different cancer types, it is important to recognize all traits

are not shared and, in some cases, certain hallmarks may prove advantageous for treatment. For instance, a pro-inflammatory response in some tumor settings may favor immunotherapy. This describes the challenges in navigating the tumor microenvironment as it can be dependent on the type of cancer, resulting in different target considerations to overcome immune resistance.

1.1.3 Pediatric hematologic malignancies

Hematologic malignancies are cancers that affect the blood, bone marrow, and lymph nodes resulting in uncontrolled growth of cells located in these sites. The first hematologic malignancy was characterized by Thomas Hodgkin in the 17th century [3, 4]. This was later described as a lymphoma and referred to as Hodgkin disease in his honor. Hematological malignancies are heterogeneous due to genomic alterations including translocations, karyotypic improvements, transformations, and post-translational modifications, that result in disease onset [5]. Genetic changes are important to diagnose and classify the stage of the disease, determine the prognosis, and subsequent treatment options [3 – 5]; **Fig. 1.1**). Most hematologic malignancies can be subdivided into two groups: Lymphoblastic/lymphocytic (B or T lymphocytes) or myelogenous/myeloid (myeloid cell lineage). These two groups include various types of leukemia such as acute lymphoblastic (ALL), chronic lymphocytic (CLL), acute myeloid (AML), chronic myeloid (CML)), myeloma, and lymphomas including Hodgkin's (HL) and non-Hodgkin's (NHL).

Leukemia is the most common type of cancer in children and adolescents younger than 20 years (25.1 percent) [6, 7]. Most childhood leukemias are acute, characterized by immature blast cells. The most common subtype, ALL, consists of approximately 75% to 80% of childhood leukemia cases, whereas AML comprises approximately 15% to 20% [8]. AML is more common with age, consisting of one-third of diagnosed cases in

teenagers [8]. Both ALL and AML are heterogeneous diseases that consists of different biological subtypes made up of multiple genetically distinct variants that can lead to chemo-resistance due to these molecular alterations. By the time of diagnosis, leukemia cells have usually replaced normal bone marrow cells and spread to extramedullary sites. A key clinical feature of most hematologic malignancies, primarily CD19 or CD20 positive lymphoblastic diseases (ALL and NHL), is immune responsiveness, which is shown in the initial success of chemotherapy treatments, adoptive cell transfer, and antibody-based therapies [5]. The success of immune therapeutics exhibits the potential to harness the immune system to treat patients affected with these diseases.

1.2 Overview of B-ALL: Prognosis and treatments

1.2.1 B cell Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia is a malignant transformation of lymphoid progenitor cells in the bone marrow, often characterized by recurrent chromosomal abnormalities and genetic alterations [7]. For pediatric B-ALL cases, more than 80% of children survive without relapse after current standard of care therapies, yet survival is poor after relapse [8-10]. B cell acute lymphoblastic leukemia (B-ALL) is the most common form of ALL, made up of over 20 molecular subtypes, and affects patients of all ages, making it one of the few cancers that spans such a wide age range [11]. Each molecular subtype consists of recurring genetic alterations associated with cell-cycle regulation, kinase signaling, regulation of chromatin, and/or lymphoid development [12]. There are two subtypes based on therapeutic relevance of note: Philadelphia chromosome (Ph^+ or BCR-$ABL1^+$ and Philadelphia chromosome-like (Ph-like or BCR-ABL1-like) ALL [13]. The common mutations are in genes involved in early B-cell

development including the t(12;21)(p13;q22) encoding *ETV6-RUNX1* (*TEL-AML1*), t(1;19)(q23;p13) encoding *TCF3-PBX1* (*E2A-PBX1*), t(9;22)(q34;q11.2) resulting in formation of the "Philadelphia" chromosome (Ph) encoding *BCR-ABL1*, rearrangements of *MLL* (*KMT2A*) at 11q23 to a range of fusion partners, and rearrangement of the cytokine receptor gene *CRLF2* at the pseudoautosomal region 1 (PAR1) at Xp22.3/Yp11.3 [12]. Approximately 20% of childhood B-ALL do not have these alterations, but rather alternative sentinel genetic lesions. High hyperdiploidy and *ETV6-RUNX1* are each present in 25% to 30% of childhood ALL cases but under 3% of cases in young adults (age 21-39 years). *BCR-ABL1*–positive ALL comprises 2% to 5% of childhood vs one-quarter of adult ALL, whereas Ph-like ALL consists of 10% of children with standard-risk ALL compared to over 25% of young adults [13]. Mutations in transcription factor genes, such as *IKZF1*, are a key alteration of BCR-ABL1 ALL and can be mutated in the absence of this alteration as well. *IZKF1* is a determinant of resistance to therapies [14]. Patients diagnosed with Ph-like subtype also carry fusions involving *CSF1R*, *JAK2*, and *TYK2*, among others [15, 16]. B-ALL subtypes can also have genetic mutations of lymphoid transcription factor, *PAX5*. Mutations in *PAX5* accounts for approximately ten percent of pediatric B-ALL, with diverse *PAX5* alterations such as *ETV6* or *NOL4L* [17].

1.2.2 Current standard of care

There are several factors that determine diagnosis and risk stratification for pediatric B-ALL. One risk classification includes age and white blood cell count (WBC) at presentation based on the criteria set by the National Cancer Institute decades ago. Ages <1 or >10 years are considered at higher risk of relapse [13]. The initial WBC is also associated with higher risk with WBC greater than 50,000/μL. Based on the NCI criteria those with initial WBC less than 50,000/μL are under standard risk classification. More

recently, residual disease at the end of induction has emerged as an important risk factor for relapse.

There are three main phases of treatment for ALL patients that are newly diagnosed that consist of various drug regimens. Different types of chemotherapy and intensities depend on the risk stratification. These phases generally consist of remission induction, consolidation, and maintenance. This typically lasts for 2 – 3 years [8]. Remission induction consists of chemotherapy that lasts approximately 4 to 6 weeks. The aim of this phase of therapy is to induce complete remission for patients, which typically occurs in over 90 percent of patients. For those patients that unfortunately experience induction failure, an allogeneic bone marrow transplant is considered [18]. The general standard of care for induction consists of agents such as corticosteroids, vincristine, and asparaginase, for NCI standard risk, and includes doxorubicin or daunorubicin for high-risk patients [18]. Prednisone or dexamethasone are the typical corticosteroids used [19]. Dexamethasone has a longer half-life and penetrates the central nervous system (CNS) better, which results in improved disease control in CNS in comparison to prednisone [20]. Patients who have treatment with dexamethasone have better event-free survival, but unfortunately it is associated with considerable long-term effects, including osteonecrosis and myopathy [21]. Consolidation therapy follows remission induction that aims to eradicate any residual disease that exists after complete remission status. Consolidation lasts approximately 6 to 9 months based on length and intensity depending on protocols in which patients with higher-risk disease having longer and more intensive regimens for consolidation [22, 23]. This phase consists of different chemotherapy agents for maximum synergy and minimization of resistance. These agents consist of

chemotherapeutics not typically used during induction such as methotrexate, cyclophosphamide, and etoposide. Methotrexate is important to maintenance of systemic leukemia and consolidation therapy.

Maintenance therapy is the final treatment stage typically lasts ≥1 year and consists of antimetabolite therapy with daily mercaptopurine and weekly methotrexate with or without vincristine and steroids. [24]. It is less intensive regimen than prior chemotherapy. There is variability in mercaptopurine tolerance between patients due to metabolic differences [25]. Understanding metabolic differences is important as studies have demonstrated impaired metabolism can lead to prolonged myelosuppression, risk of infection, and treatment interruptions. Studies have focused on titrating the dose to keep the WBC lower because higher WBC increases the risk of relapse [26,27]. Thus, there is a balancing act that occurs to manage the risk of myelosuppression with severe infection and some regimens include vincristine and steroids [28].

One other component of treatment is targeted against CNS disease. This includes treatment at diagnosis and prophylaxis. CNS directed therapy against the CNS disease is important since most children will develop CNS relapse without specific therapy at this site. There are various methods of eradicating leukemia from the CNS, which includes CNS directed radiation as prophylaxis (which prevented CNS relapse but has severe lasting effects including neurocognitive and musculoskeletal growth), direct intrathecal administration of chemotherapy, particularly methotrexate or a combination of methotrexate, cytarabine and hydrocortisone, and systemic administration of chemotherapy that can cross the blood-brain barrier [29].

Hematopoietic stem cell transplantation (HSCT) is generally a line of treatment for pediatric patients that experience refractory disease, or early bone marrow relapse within the first 36 months of remission [30]. High non-relapse mortality outcomes in earlier HSCT attempts resulted in identifying and understanding graft-vs-host disease (GVHD). Yet, it was reported that patients with mild GVHD exhibited lower relapse rates compared to those with no evidence of GVHD [31]. This suggests the importance of graft vs leukemia for the HSCT success and contributed to new exploration of other ways to engage the immune system against B-cell malignancies.

1.2.3 Challenges with current standards of care

Strides have been made in chemotherapeutics and supportive care that have resulted in a significantly improved survival of children with B-ALL. Yet, there are still patients with relapsed and refractory disease that have a poor prognosis. HSCT was one of the initial immune-therapeutics used for the treatment of patients with relapsed or refractory disease, yet other novel therapies such as bispecific antibodies (T-cell engagers) and chimeric antigen receptor T-cells (CAR-T) therapy are novel FDA-approved therapeutic options now for these patients. With several immunotherapeutic agents in development a new era of frontline treatment is emerging for addressing relapsed/refractory disease. This may portend the potential for immunotherapies to become the frontline of treatment regimen for patients.

1.2.4 Immunotherapies

There are several antibody-based therapies that are targeted at certain tumor antigens. These are typically monoclonal antibodies, antibody-drug conjugates, or bispecific antibodies. Monoclonal antibodies that target tumor surface antigens currently

used in clinic include rituximab, which is an anti-CD20 antibody and dinituximab (anti-GD2 antibody) [32,33]. Both monoclonal antibodies work by binding to tumor cells that marks them for destruction by immune cells [34]. Further, drug-antibody conjugates target the antigen binding domain (Fab) of tumor cells, leading to antibody-dependent cytotoxicity (ADCC). These therapeutics include anti-CD22/calicheamicin conjugate inotuzumab ozogamicin [35]. CD22 is involved both activation and regulation of B cells and is expressed in the B ALL cells of the majority of patients [36,37]. Unfortunately, allergic reaction and immune reactivity against the monoclonal antibody are some limitations to this type of approach.

Additionally, blinatumomab, a bispecific T-cell engager, consists of both two single-chain variable antibody fragments (scFv). One scFv binds to CD19 which is expressed on B leukemia cells and then the other binds to the T-cell receptor/CD3 complex (**Fig. 1.2**). This triggers T-cell induced apoptosis of any CD19 positive leukemia cells, creating an immunostimulatory T-cell profile [38]. CD19 is a marker on most B cell malignancies and is specific to B cells, making it an ideal target in immunotherapy for these cancers. Linking together T-cells and the tumor cells catalyze the formation of the immunologic synapse and result in a polyclonal T cell response and cytotoxicity of the tumor cell. This process is independent of MHC expression, bypassing mechanisms of immune evasion. In a phase 2 study in patients with relapsed/refractory Philadelphia chromosome (Ph)-negative B cell ALL has been shown to be effective, with half of these patients were able to proceed with allogeneic stem cell transplantation, and 28% of individuals had an overall survival of (OS) ≥30 months [39, 40].

Chimeric antigen receptor (CAR T) therapy has also been a promising immunotherapeutic for hematologic malignancies. It involves not only targeting specific tumor antigens but also targets immune effectors to elicit an anti-tumor response. CAR T-cells involve engineering autologous T-cells to express chimeric antigen receptors against a specific tumor surface antigen. CAR T-cells are both HLA independent and antigen specific and are independent of MHC expression as are BiTEs. The general structure consists of a single-chain variable fragment with a linked hinge region and transmembrane domain for intracellular T-cells signaling with a costimulatory domain [41]. T-cells are acquired via leukapheresis from patients and then the CAR expressing T-cells are subsequently expanded [41]. Engagement of tumor antigen by the CAR leads to T-cell cytolytic activity and proliferation. The first line of successful CAR T therapeutics has targeted CD19 B cell malignancies. Responses in ALL patients has been successful in heavily pretreated relapsed, and refractory patients [42,43]. This demonstrates the efficacy of immunotherapeutic in the context of ALL.

Further, immune checkpoint inhibitors related to the regulation of T-cell homeostasis have been used to regulate both stimulatory and inhibitory checkpoints. These include cytotoxic T-lymphocyte antigen 4 (CTLA4), programmed cell death 1 (PD1) and its ligand programmed cell death ligand 1 (PD-L1). These markers all inhibit T-cell activity. Thus, antibodies targeted against these antigens enhance T-cell activity creating a more immunostimulatory environment [44]. Unfortunately to-date these have not been as effective in hematological malignancies as compared to solid tumors.

Antibodies specifically bind to tumor antigens that enhances the efficacy of these targeted therapies compared to chemotherapy. Unfortunately, there are still disadvantages including downregulation of antigens in leukemia that escape recognition and destruction. HSCT has been an accepted standard of care for pediatric patients that undergo relapsed/refractory disease, yet it poses challenges as it is associated with morbidity including infections and graft-vs-host disease (GVHD) in addition to difficulty identifying HLA-matched donors. Based on data from the Center for International Blood and Marrow Transplant Research (CIBMTR), three-year survival outcomes from AML patients who undergo allogeneic HCT, is 70% compared to 76% in patient with ALL [45]. These outcomes drop dramatically in AML and ALL patients with advanced disease (30% and 48%, respectively) [45].

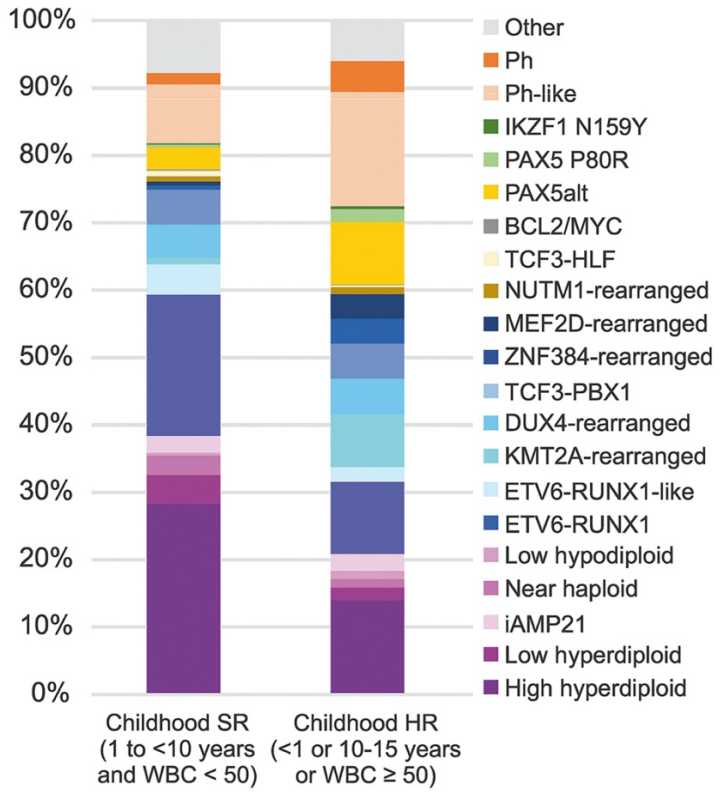


Figure 1.1. Distribution of different genetic variations in pediatric B-ALL. Adapted from Pediatric acute lymphoblastic leukemia, *Haemotologica*, 2020. (SR - standard risk, HR – high-risk, and WBC - white blood cell count).

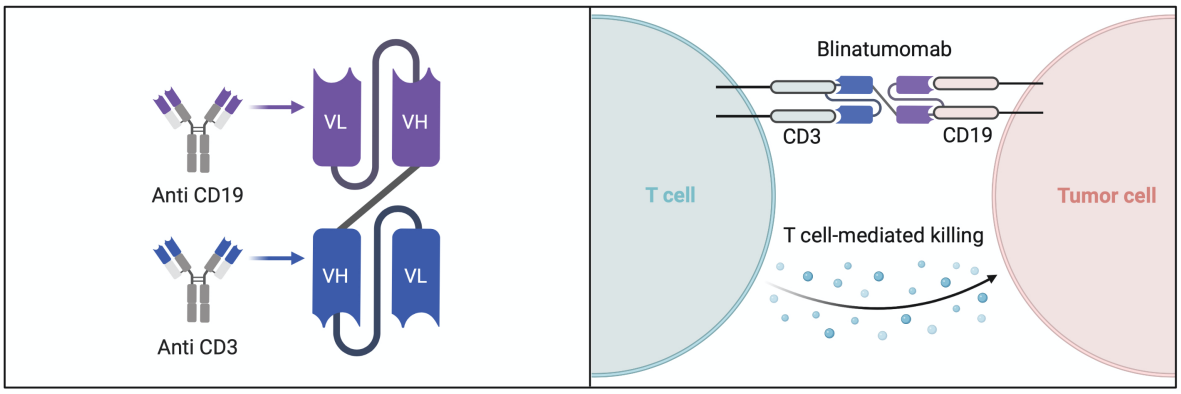


Figure 1.2. Schematic of blinatumomab, bispecific T-cell engager. Created with Biorender.

Chapter 2

Overcoming mechanisms of immune evasion in B-ALL

2.1.1 Immune evasion mechanisms in cancer

The immune system consists of various types of immune cells including dendritic cells, natural killer (NK) cells, T and B cells. There are two arms of the immune system – the innate and the adaptive immune system. The innate immune response consists of a non-specific immune response, including damage-associated molecular patterns (DAMPs) and Toll-like receptor (TLR) ligands, which activate cytokine release [46]. Both B cells (responsible for antibody production) and antigen presenting cells (APCs) that stimulate $CD4^+$ T-helper cells are a part of the innate immune response that promote adaptive immune responses. Helper T-cells stimulate cytotoxic T lymphocytes (CTL) cells that eliminate pathogens and result in immune memory cells [47]. Prevention of tumors occurs due to the ability of cytotoxic $CD8^+$ T-cells to eliminate these abnormal cells [48]. Tumor cells have different biochemical and antigenic characteristics from healthy cells and have acquired mechanisms of immune evasion (**Fig. 2.1**). Thus, CTLs recognize tumor cells but often cannot control tumor growth [49]. CTL dysfunction is induced by continuous stimulation by tumor antigens and immunosuppressive tumor microenvironment (TME), which can drive T cell functional depletion and cancer progression [50].

The concept of immune editing details the interaction between the immune system and the establishment of cancer. CTLs recognize tumor antigens and promote the removal of tumors. This process selects cancer cells with mutations that provide resistance to immune effectors and tumor cell survival benefits in the microenvironment [51]. Immune editing consists of elimination, equilibrium, and avoidance [52]. In the elimination phase, the immune system recognizes, and kills transformed cells and new tumors through

antibody production. This process typically starts with macrophages, dendritic cells, and infiltrating lymphocytes including NK T-cells migrating to the tumor site [53]. In addition, interferon-gamma (IFNγ) and interleukin 12 (IL-12) enhance the cytotoxic response of NK and CTL cells and promote tumor death through apoptosis [54]. Both T-cell subsets infiltrate the tumor site upon identification of tumor antigens. Unfortunately, not all cancer cells are eradicated during this phase resulting in either dormancy or equilibrium (**Fig. 2.1**) [55]. Equilibrium consists of cancer that is clinically undetected, suggesting that tumor cells adapted to coexist with the immune system for up to several years [56]. Tumor escape and recurrence occurs with the loss of tumor antigen, upregulation of PD-L1, establishment of Tregs, and myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment [57; **Fig 2.1**]. Thus, escape consists of cells being able to proliferate and avoid immune detection in this manner.

Hijacking of the immune system can occur with either gain-of-function mutations, induced aberrant HLA expression or dysregulation of antigen processing mechanisms [58]. Alterations in both PD-1 and PD-L1 expression in both tumor and normal cells also inhibit activation of not only T-cells, but other immune cell populations, promoting further tumor immune escape [59]. Chronic PD-L1 expression, primarily by TAMs, prolongs tumor specific T-cells inducing an immunosuppressive tumor microenvironment [60]. NK cell function and NK receptors can be altered resulting in the release of immunosuppressive cytokines like IL-10 and transforming growth factor beta (TGFβ). Thus, the immune system promotes tumor progression via these mechanisms.

2.1.2 Mechanisms of immune evasion in B-ALL

Hematological cancers are typically poorly immunogenic or fail to alert innate or adaptive immune mechanism. They are adept at immune evasion as a result. Acute leukemias spread rapidly upon occurrence which negatively impacts anti-leukemia immunity. ALL immune tolerance has been found to be due to the lack of expression of co-stimulatory molecules such as CD80, exhibiting poor T-cell activation [61, 62]. Low mutational burden that exists in ALL also results in low immunogenicity [63, 64]. B-ALL cells no longer function as antigen-presenting cells (APC), resulting in its rapid spread that affects anti-leukemia efforts due to non-activated T-cells. This in turn promotes an immune suppressive microenvironment [65]. Previous studies demonstrate anergic T-cells are the result of interleukin-10 (IL-10) expression induced by CD40 activation and abnormalities in IL-12 and CD40 have been identified in patients with ALL [66, 67].

There are several immune tolerance mechanisms that cancer utilizes to escape immune detection including metabolic modulation, immune suppressive cytokines, expansion of MDSCs and Tregs, and inhibitory ligands such as PD-L1 [68]. Many studies on ALL exhibit defective antigen presentation of MHC-I molecules and HLA class I and II expression [69, 70]. Further, mechanisms that disrupt immune checkpoint expression and changes in the balance of pro- or anti- inflammatory cytokines are reasons ALL evades immune surveillance [67, 71]. These mechanisms will further be described below.

One of the primary mechanisms different types of cancer use to avoid immune detection is by overexpressing co-inhibitory ligands. Cell surface molecules including cytotoxic-T-lymphocyte associate protein 4 (CTLA-4), PD1, and PD-L1 are typical co-

stimulatory or inhibitory signals that maintain T-cell immune homeostasis [72]. CTLA-4 typically halts T-cell activation triggering inhibition within the T-cell to avoid prolonged or overactivation [73]. CTLA-4 expression is elevated in T-cells in patients with high-risk ALL and it is elevated in the serum in about 70% of B-ALL pediatric patients with active disease [74, 75]. It is also associated with poor prognosis in pediatric patients. High surface CTLA-4 expression on immune cells have been detected in patients with B-ALL who died from the disease [75]. Thus, it is possible CTLA-4 expression from ALL cells could be a potential mechanism of immune evasion.

Additionally, both PD-1 and PD-L1, inhibitory immune checkpoints that suppress T-cells, overexpression occur in numerous types of cancer that evade the immune system [76, 77]. PD-1 expression has been reported to inhibit CTL function observed in patients with AML [77]. CTLA-4 and PD-1 expression in hematological malignancies are reported as potential immune evasion methods, promoting leukemogenesis [78]. Abnormal expression of PD-1 has been identified in bone marrow biopsies in the pediatric ALL patients' T-cells [79]. Pediatric ALL blasts have increased expression of PD-L1 in patients who unfortunately relapse with ALL [80].

Additional checkpoints include T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), inducible T cell costimulatory COS (CD278) and lymphocyte-activation gene 3 (LAG-3) that are involved in T-cell suppression. TIM-3 is associated with apoptosis, and Tregs that upregulate this surface marker have higher suppressive features compared to negative TIM3 Tregs [81]. Expression of LAG-3 is associated with immunosuppressive Tregs correlated with an increase in IL-10 secretion

by this cell population [82, 83]. Further, both TIM-3 and LAG-3 are expressed in patients with ALL [81].

Differences in anti-inflammatory (IL-10, IL-13, and TGF-β) and pro-inflammatory cytokines (IL-1β, IL-6, IFN-α, and TNF-α) contribute to immune evasion in ALL [84; **Fig 2.2**]. Inflammatory cytokine signatures including IL-8, IL-6 and IL-18 have been found in children diagnosed with pre-B ALL [84]. In ALL, the bone marrow (BM) microenvironment is overall pro-inflammatory and leukemia cells express IL-10 and TGF-β leading to reduced immunogenicity [85]. The pro-inflammatory BM micro-environment results from secretion from hematopoietic and stromal cells, yet cancer cells have been shown to dampen immune activation by creating an anti-inflammatory environment, blocking pro-inflammatory cytokines to evade immune detection [86]. Immune cell dysfunction also occurs because of TNF-α secretion by B-ALL [87]. IFN- γ and interleukin 6 (IL-6) are two of the most prominent cytokines linked to cancer immunity [88]. In patients with ALL, IFN-γ expression is low, implying that the immune system is suppressed, and leukemia cells are avoiding immune monitoring [89]. Abnormal IL-6 expression is also present in ALL cases, exhibiting its important role in leukemia in this malignant disease [88]. Additionally, cytokines and chemokines that are associated with immunotolerance include IL-1, IL-7, CCL2, CXC-10, and CXCL-12 [90]. These alterations in the anti-inflammatory and pro-inflammatory cytokine axis are relevant players to consider in oncogenic processes and the development of cancer. Thus, the ratio of both pro- and anti-inflammatory cytokines signatures, as well as which specific inflammatory mediators are present at various points of presentation in the BM environment is a significant consideration for therapeutic efficacy.

Another important mechanism for immune evasion in leukemia is the abnormal proliferation of immune cell populations including T-cells, NK cells, and MDSCs. There are two distinct subsets of T-cells are involved CTLs that eradicate cancer cells and T helper cells required for APC activation and proliferation. Tregs ($CD4^+CD25^+$ $Foxp3^+$) play a role in tumor development and progression by suppressing anti-tumor immunity in the tumor microenvironment [91]. Tregs play an important role in self-tolerance and immune homeostasis processes under physiological conditions by suppressing normal and pathological immune responses [92].

There are other lymphoid and myeloid cells that compose the BM compartment including include NK, MDSCs, and macrophages have also been implicated in immune suppression. Recent studies have identified a role of NK cells in the initiation and development of ALL [93, 94]. Further, NK cells in patients recently diagnosed with ALL exhibit an abnormal NK ligand expression resulting in lack of function [94]. There are also differences that exist in NK cells in ALL subtypes, in which patients who have BCR-ABL^+ ALL have higher expression of NKG2D and DNAM1, two activating ligands expressed on NK cells patients [95]. Decreased NK cell function and numbers in the peripheral blood are considered the most significant immune surveillance issues that arise in ALL [96]. Investigations are being conducted to identify how to leverage NK cells as a potential therapeutic in ALL treatment.

MDSCs that consists of immature monocytes or granulocytes also contribute to immune suppression. There are two main subpopulations, monocytic MDSCs (MO-MDSCs) and polymorphonuclear MDSCS (PMN-MDSCs) that suppress activation, proliferation and cytotoxicity of effector T and NK cells [97, 98]. There is still more research to be discovered on the role of MDSCs. Yet, a high number of MDSCs was

associated with patients diagnosed with ALL and is also associated with blast cells in the BM of ALL patients [99]. MDSCs also secrete immunosuppressive cytokines, TGF-β and IL-10, that induce Tregs and T-cell suppression in the BM microenvironment [100].

Another population associated with immune suppression are macrophages. There are two subtypes including M1 (associated with antitumor effects) and M2 (associated with immune suppressive effects) [101]. The M1 macrophage population is reduced in B-ALL adult patients with subsequent increases in M2 macrophages [79]. Tumor-associated macrophages (TAMs), which are an M2 subtype, are associated with immunosuppression in ALL [102]. Additionally, splenic macrophages were shown to have a M2-like phenotype and secrete immunosuppressive cytokines such as IL-10 and TGF-β in murine leukemia models [103].

Although improved treatments have led to better patient outcomes, immune evasion by ALL cells remains an area of investigation. The identification of leukemic cell strategies, such as increasing cytolytic T cell response using BITEs or CAR T therapy, are potential immune therapeutic strategies to overcome immune evasion and immunosuppression in the ALL microenvironment. Further research describing immune evasion mechanisms in ALL development and progression are needed to gain further insight into both molecular and cellular mechanisms of leukemia progression. This can contribute to the development of novel immune therapies for ALL. Current immunotherapeutic strategies for ALL are described below.

2.1.3 Current immunotherapeutic strategies to overcome immune evasion in B-ALL

Induction, consolidation, and long-term maintenance therapy comprise the first stage of ALL treatment as previously described. The foundation of ALL therapy is

chemotherapy, which has significantly improved survival in ALL [7, 8]. Unfortunately, approximately 20% of patients relapse and there is a need to improve overall survival rates [7,8]. Currently, there are several immunotherapeutic strategies that are becoming standard of care for selected patients to overcome immune evasion in B-ALL. These include monoclonal antibodies (mAbs), immune checkpoint inhibitors, CAR T-cells, and bispecific T-cell engagers (BITEs) [104 – 108].

BITEs have been explored due to their ability to link both T-cells and leukemia cells to lead to a favorable immune response [104]. In ALL, Blinatumomab (anti-CD19/anti-CD3) binds to $CD3^+$ T-cells and CD19+ B lymphocytes [104]. It is administered as a 4-week continuous infusion due to its short *in vivo* half-life [108]. Initial adult phase II studies in relapsed/refractory (R/R) B-ALL showed favorable results with rates of complete remission with or without complete hematologic recovery (CR/CRh) of 69% (25 out of 36) and 43% (81 out of 189), respectively [108]. Further, in younger populations, the Children's Oncology Group has explored the use of blinatumomab in clinical trials in pediatric B-ALL patients. AALL1121, a phase I/II study conducted by COG and the I BFM Study Group, enrolled 70 patients with relapsed/refractory B-ALL [109]. Among patients receiving the recommended dose of blinatumomab 39% achieved CR within two cycles. In patients with relapsed disease only, 48% CR was achieved [109]. Due to the unfavorable prognosis of this group (> 70% of patients had relapsed within 6 months of the previous treatment), these results are promising. Given the anti-leukemia activity of blinatumomab in addition to its efficacy in patients with MRD and tolerability, the U.S. Food and Drug Administration (FDA) has now approved blinatumomab for treatment of Ph-R/R B-ALL in 2016 for children and adults [108].

Additionally, CAR T-cells act independently of HLA recognition which may prove beneficial in ALL cells that express HLA at lower levels. A CAR consists of an extracellular scFvc which identifies the tumor antigen fused to a transmembrane domain, with intracellular activating/co-stimulation molecules such as motives CD3ζ, CD28, and 4-1BB, and it is transduced to T-cells promoting a cytolytic effect [108 – 111]. CAR T-cells are currently FDA- approved for both treatment of leukemia and lymphoma in patients [112]. A phase I/IIA investigation for CAR modified T cell CTL019 therapy for ALL relapsed patients who had undergone allogeneic hematopoietic cell transplantation demonstrated durable remission rates after 24 months, with 78% OS and a 6-month event-free survival (EFS) rate of 68% [42]. However, in a group of refractory/relapsed patients with B cell ALL, CD22 CAR T cell therapy treatment resulted 70.5% complete remission compared to previously treated CD19 CAR T-cells without success [111, 112]. One of the potential benefits of CAR T-cells is the ability to generate a CAR that can target different types of antigens (such as CD19, CD20, and CD22) [111, 112]. Limitations include challenges with expansion and persistence *in vivo* in addition to cross reactivity and cytokine release syndrome (an onset of symptoms including fever and hypotension caused by the release cytokines by T-cells) [106].

Adoptive T cell therapy (ACT) is focused on expanding TILs and infusing in patients after depletion of lymphocytes. This therapy produces an anti-tumor immune response through infusion of modified T-cells *ex vivo*. ALL is typically associated with a low mutational load, which could be possibly overcome by developing neoepitope-CD8+ T-cells to treat patients with ALL [113]. The co-culture of HLA-specific APCs with neoepitopes and isolated CD8+ tumor-infiltrating lymphocytes results in TNF-α and IFN-

γ production. This could be potential method for immunotherapy in leukemia for use in the consolidation phase or as a treatment [63].

2.2 IL-12

2.2.1 IL-12 overview

IL-12 (**Fig. 2.3**) is a pro-inflammatory cytokine that is an important player in the regulation of T-cell responses, and has great antitumor efficacy. IL-12 consists of a heterodimer with p35 and p40 subunits that form a bioactive IL-12p70 with a molecular weight of 70 kDa [114 – 117]. Cells of the innate immune system including monocytes, macrophages, and dendritic cells secrete IL- 12 in response to infections [118]. The potency of IL-12 is enhanced by other cytokines such as interferon-γ (IFN-γ), IL-15 or CD40L–CD40 cell–cell interactions [119 – 121]. IL-12 is a regulated negatively by cytokines including IL-10 and TGF-β1 [122 – 123]. The heterodimeric IL-12 receptor (IL-12R) that consists of IL-12Rβ1 and IL-12Rβ2, is responsible for IL-12 sensing and downstream effects in target cells [124]. For the development of high-affinity IL-12 binding sites, both receptor subunits must be co-expressed. The IL-12R complex has been discovered on NK cells, NK T-cells, and activated T-cells as well as myeloid cell types [125, 126]. IL-12Rβ1 is expressed by naive T-cells but not IL-12Rβ2, which is required for signal transduction downstream of the receptor complex [127]. Both IL-12 receptor chains are generated when T-cells are activated by the T-cell receptor, which is further boosted by IL-12, IFN- γ, tumor necrosis factor- α (TNF- α), and anti-CD28 co-stimulation [128, 129]. The JAK-STAT (Janus kinase–signal transducer and activator of transcription) pathway is activated when the receptor is successfully triggered resulting in STAT4 phosphorylation and subsequent induction of cytokines and cytotoxic molecules that result in an anti-tumor immune response [130,131].

By connecting innate and adaptive immune responses, IL-12 plays a crucial role in the regulation of inflammation. By promoting the production of cytokines and cytolytic factors like perforin and granzyme B, IL-12 released by APCs enhances the activation and proliferation of NK and T-cells, as well as their effector capabilities [132 – 134]. Furthermore, IL-12 converts T-cells into type 1 helper T (Th1) effector cells [135 – 136]. Th1 polarization is exacerbated by IL-12 preventing the differentiation of regulatory T-cells and Th17 cells by secretion of TGF-*β,* as well as its inhibition of the developmental program of type 2 helper T-cells [137]. IL-12 also induces an optimal effector memory pool of T-cells and T follicular helper cells [138, 139]. IL-12 receptor activation does not engage the canonical STAT pathway in APCs, yet it improves APC presentation of weak immunogenic tumor peptides [140, 141]. IFN-γ, which is released in response to IL-12 stimulation alone or in combination with synergizing factors such as IL-2 and IL-18, is a key mediator of IL-12-induced responses [142, 143]. In a positive feedback loop, IFN-γ works on APCs to trigger or boost IL-12 release aside from IFN-γ. IL-12 also stimulates the production of TNF-α, GM-CSF, and IL-2 [144]. IL-12 also enhances interactions between T-cells and DCs through CCL1 and CCL17 production, capable of augmenting the efficacy of DC vaccinations [145]. Taken this data in consideration, IL-12 has the potential to be used as an immune therapeutic in ALL.

2.2.2 Preclinical and clinical models of IL-12 treatment

The therapeutic effects of IL-12 have been evaluated extensively in various preclinical cancer models, mainly delivered intravenously, intraperitoneally, or intratumorally. The anti-tumor effectiveness of IL-12 is both dose and context dependent and has been shown to reduce tumor growth via $CD8^+$ T-cells and NK cells stimulation [146]. IL-12 administration in *in vivo* models has typically occurred through direct

infusion of the recombinant protein, by gene therapy using viral and non-viral vectors, electroporation, by IL-12-containing microspheres and nanoparticles or by the transfer of IL-12-overexpressing stromal and immune cell types. Unfortunately, in clinical studies it has not been well tolerated [147, 148]. For example, in one phase II trial 12 out of 17 enrolled patients experienced severe side effects and unfortunately the death of two patients [149]. Patients were given a maximal dose of 0.4 ug/kg per day which was found to be previously tolerated in a phase I study, but unfortunately a change in dosing schedule proved intolerable. Due to severe toxicities based on dosing and disappointing clinical responses in phase 2 studies this raised the question if IL-12 was as effective in humans as observed in mice. However, due to the strong immune responses in patients resulting in toxicity, it is evident it does have potent activity in humans. Further, limited efficacy may be due to ineffective delivery of IL-12 to reach the tumor microenvironment. Thus, ideal targets include for IL-12 immunotherapy are not only those lymphocytes in circulation but in the tumor microenvironment or activated T-cells, NK cells, TAMs and MDSCs.

IL-12's high anti-cancer effect make it a good choice for use in combination with other therapies aimed at increasing the tumor's immunogenicity. In this regard, there have been different IL-12 administration strategies (**Fig. 2.4**) and combination therapy of IL-12 with cytokines, peptide vaccines, chemotherapeutic drugs and monoclonal antibodies enhanced the cytokine's therapeutic effectiveness in melanoma, bladder carcinoma, and mammary carcinoma tumor models [152 – 155]. Although combining cytokine therapies would stimulate immune responses, this results in high systemic levels of IFN-γ production [156]. Regarding combination with chemotherapy, enhanced antitumor effects were only observed if IL-12 was administered early after chemotherapy

[153]. This shows the importance of dose scheduling for immune intervention in chemotherapy-induced antitumor responses [153]. One such effective combinatorial treatment with IL-12 was shown in human epidermal growth factor receptor (HER)-2/neu transgenic mice in which mice treated with IL-12 in combination with tamoxifen or HER-2/neu multi-peptide vaccines experienced no tumor progression. [157]. Co-administration of IL-12 and anti-HER-2 antibody, trastuzumab, in colon adenocarcinoma led to tumor regression [154]. Based on these results, combining IL-12 with other therapeutics has a lot of promise for overcoming tumor-associated immune suppression.

2.3 Rationale for studying IL-12 as an immunotherapeutic for B-ALL

The overarching aim for this dissertation research was to understand the mechanisms of immune evasion in B-ALL, particularly with respect to alterations in the tumor immune microenvironment and determine how IL-12 overcomes these mechanisms. Additionally, developing strategies to maximize IL-12 delivery to the tumor microenvironment are of increasing interest. Understanding the various mechanisms in which IL-12 provides anti-leukemia immunity is important for future clinical context. Thus, the next chapter (chapter three) aims to address the impact of IL-12 in a genetically modified B-ALL cell line that secretes IL-12. This chapter described the discovery of IL-12 in clearing leukemia burden in immune-competent mice as early as day 7. IL-12 treatment of mice exhibited efficient T-cell activation in part due to increased antigen presenting cells (APCs) and the ability to clear leukemia even upon re-challenge of an aggressive B-ALL cell line. These studies demonstrate that IL-12 elicits a robust, sustained T-cell response.

However, the impact of IL-12 in the B-ALL bone marrow microenvironment, specifically in T-cells, and subsequently potential combination treatment considerations in B-ALL have not been elucidated. Thus, chapter four aims to address a mechanism of T-cell suppression by B-ALL and characterize the impact of IL-12 in the immune suppressive B-ALL microenvironment.

The ultimate goal of this study is to leverage IL-12 as an immunotherapy in B-ALL and develop targeted treatment strategies to which are well-tolerated in pediatric patients with hematological malignancies.

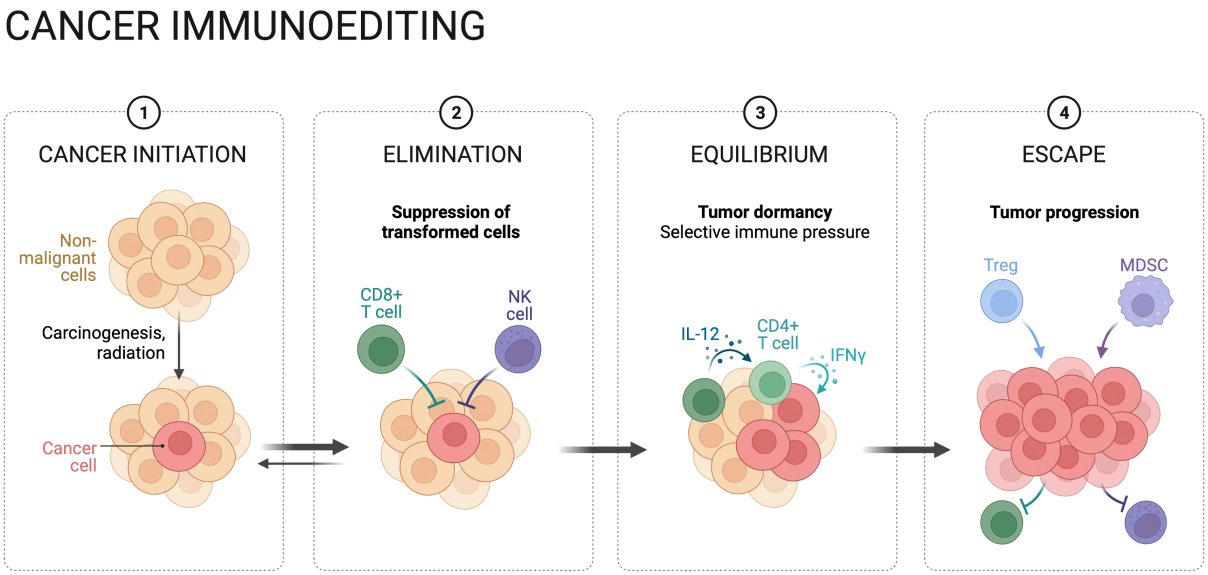


Figure 2.1. The four stages of cancer immunoediting. Created with Biorender.

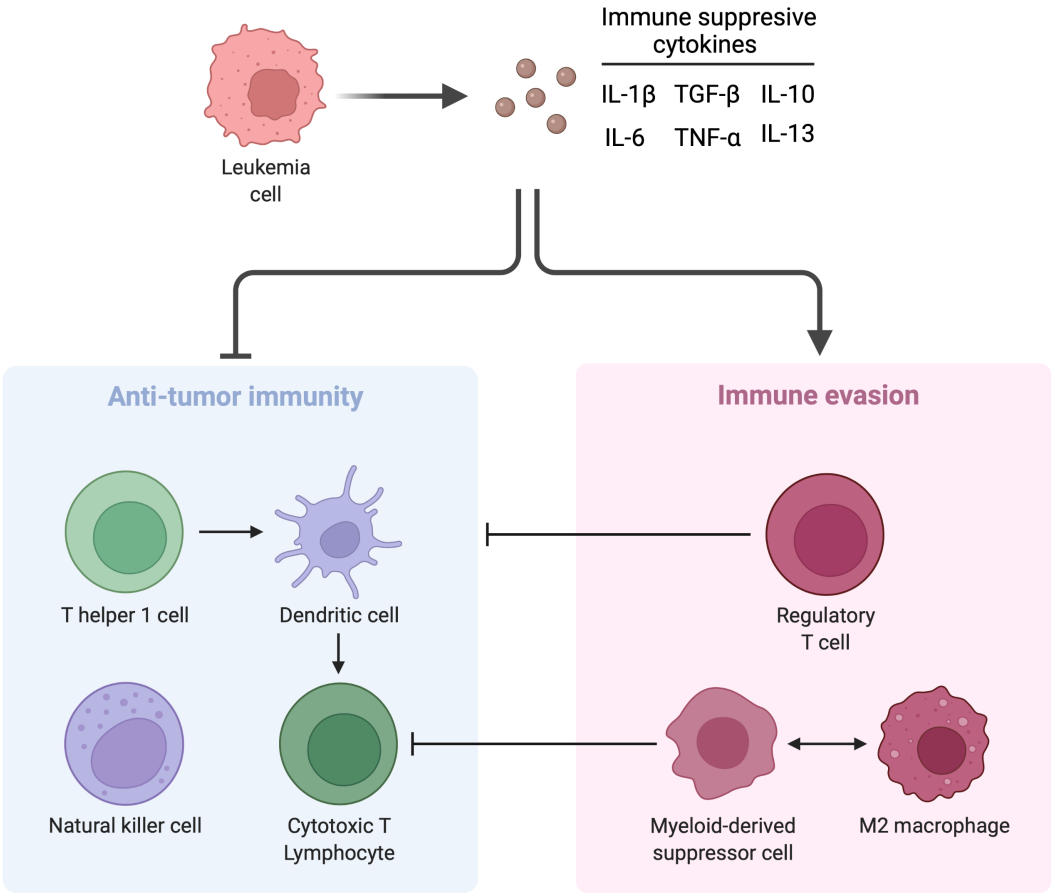


Figure 2.2. Immune suppressive effects of cytokines in leukemia. Created with Biorender.

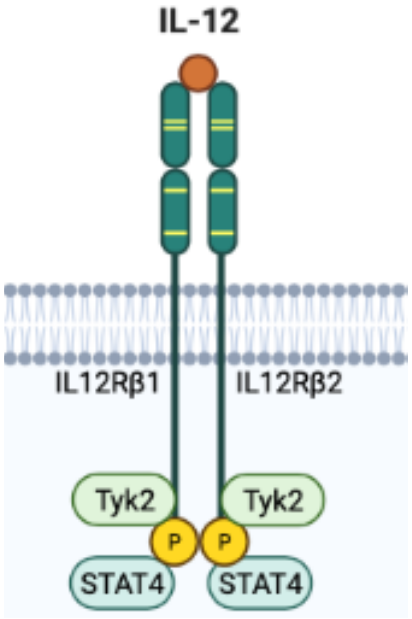


Figure 2.3. IL-12 schematic. Created with Biorender.

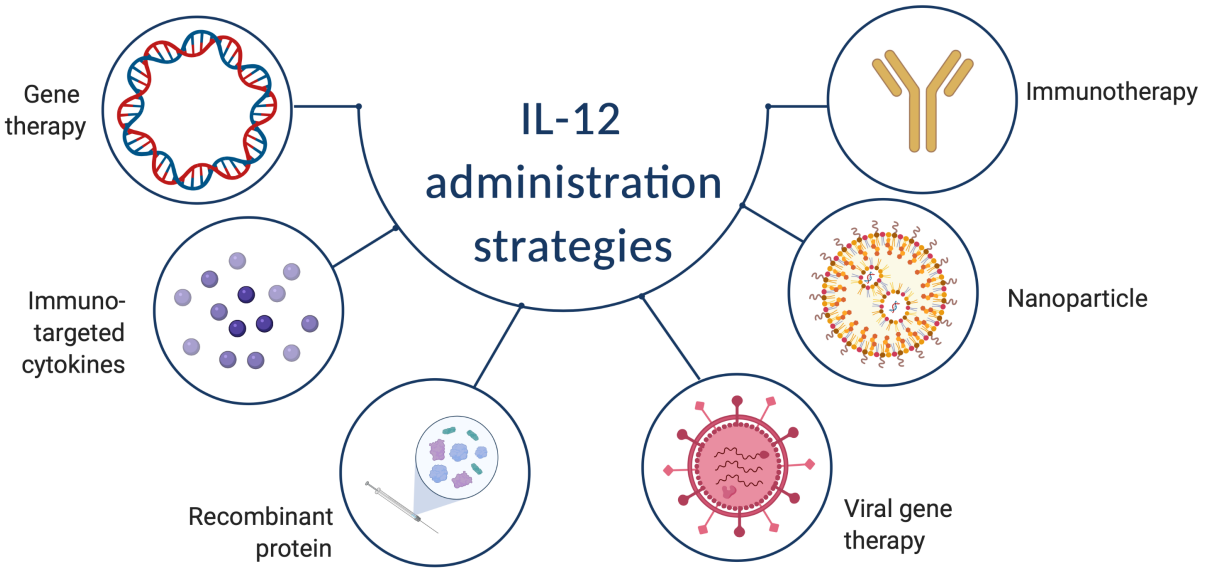


Figure 2.4. Therapeutic strategies for IL-12 administration. Created with Biorender.

Chapter 3

IL-12 induced leukemia remission is dependent upon both CD4 and CD8 T-cells

Portions of this chapter are published in Rabe JL, Gardner L, **Hunter R**, et al. IL12 Abrogates Calcineurin-Dependent Immune Evasion during Leukemia Progression. *Cancer Res*. 2019 Jul 15;79(14):3702-3713. PMID: 31142509. RH completed *in vivo* experiments pertaining to $CD4^+$ and $CD8^+$ T-cells.

3.1 Introduction

Calcineurin is a serine/threonine phosphatase that has previously been extensively studied in the context of T-cell activation. Yet, research studies also identified calcineurin plays a role oncogenesis and drug resistance in leukemia and lymphoma [159 - 162]. Thus, when identifying the role of calcineurin in leukemia cells, we made a novel discovery that leukemia cells deficient in calcineurin secreted high levels of IL-12. This resulted in leukemia clearance and long-term survival in mice engrafted with these cells compared mice engrafted with Cn-expressing leukemia cells. Thus, the initial aim of my thesis sought to explore relevant immune cells acted upon by IL-12. I demonstrated NK cells were not responsible for leukemia clearance in this model. However, $CSF1R^{+}$ myeloid cells and T-cells were found to be significant for long-term survival of mice. Examining the role of IL-12p40 in the bone marrow microenvironment during leukemogenesis demonstrated there were significant differences in immune cells subsets, with recipients of calcineurin-deficient leukemia (shCnB) having a higher number of T-cells compared to control recipients. This demonstrates IL-12 secretion elicits APCs to activate T-cells resulting in leukemia clearance in the BM microenvironment.

3.2 Background

We previously demonstrated the clearance of leukemia occurred in wild-type mice with an intact adaptive immune system, but not in *Tcra*$^{-/-}$ mice deficient in mature T-cells. WT mice were engrafted with either GFP-expressing control or calcineurin-deficient leukemia (shCnB) and bone marrow was harvested seven days later to determine the role of T-cells in leukemogenesis. As controls, a group of mice free of leukemia was used. At day 7, bone marrow was isolated from each of the three groups of mice. When shCnB

leukemia recipients were compared to control recipients, the percentage of $CD3^+$ T-cells in the bone marrow was higher in the shCnB recipients (**Fig. 3.1A**). $CD8^+$ T-cell subsets were found to be more abundant in mice engrafted with shCnB mice recipient compared to control recipients (**Fig. 3.1A**).

Further, investigating the differential expression of immune signaling molecules, most cytokines and chemokines were found in higher concentrations in the bone marrow of shCnB leukemia recipients than in the non-silenced (shNS) control recipients. In validating these findings via an ELISA, it was confirmed that shCnB leukemia cells secreted significantly more IL-12 than controls (**Fig. 3.2A**). Furthermore, we found that shCnB cell supernatant increased T-cell activation compared to shNS cell supernatant, as measured by intracellular IFN-γ and TNF-α production as shown in $CD8^+$ T-cells (**Fig. 3.2B**).

We next identified if higher levels of IL-12 are sufficient to slow the progression B-ALL *in vivo*. Both immune-competent and immune-deficient recipients of parental luciferase-expressing BCR-$ABL1^+$ B ALL cells were treated with recombinant IL-12 (rIL-12). Wild-type mice treated with rIL-12 demonstrated a reduction in leukemia burden after 4 days of treatment and entered a durable remission with prolonged survival and no evidence of disease 3 weeks after completion of treatment (**Fig. 3.3A, B**). Mice that were immune competent recipients of BCR-$ABL1^+$ B-ALL cells and rIL-12 were re-challenged with leukemia cells 60 days after the first injection, and all mice survived past 30 days without evidence of disease (**Fig. 3.3B**). Further, *Rag1*$^{-/-}$ mice treated with rIL-12 demonstrated disease burden reduction after treatment initiation and prolonged survival

with no intact adaptive immune system but did relapse after treatment was discontinued (**Fig. 3.3A, B**). This demonstrates a role of the innate immune system in IL-12 induced leukemia clearance.

Taken these data into account, my initial studies aimed to characterize the immune microenvironment based on this immunogenic model of leukemia previously shown to secrete high levels of IL-12 due to calcineurin knockdown [158]. Thus, in the earlier investigations for my thesis I sought to identify immune cell subsets responsible for leukemia clearance in the presence of IL-12 secretion.

3.3 Materials and Methods

Mice

C57BL/6, Tcrα$^{-/-}$ (B6.129S2-Tcra^{tm1Mom}/J) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in micro-isolators in standard conditions in the Division of Animal Resources Facility in the Health Sciences Research Building at Emory University. All animal studies were approved by the Emory University Institutional Animal Care and Use Committee.

Leukemia Model

The luciferase expressing, BCR-ABL1^{+} Arf$^{-/-}$ B-cell acute lymphoblastic leukemia line was originally provided by Dr. Richard Williams [163 – 167]. Leukemia cells were transduced with lentiviruses expressing non-silencing control shRNA (shNS) or shRNA against *Ppp3r1*, which encodes the essential regulatory subunit of calcineurin (shCnB),

with over 90% knockdown as previously described [164]. A total of 5×10^5 cells were transferred via tail vein injection into un-irradiated, 6–8-week-old, female wild type (WT) or $Rag1^{-/-}$ recipients. After intraperitoneal injection of luciferin and anesthesia with inhaled isoflurane, leukemia burden was measured by the In Vivo Imaging System (IVIS) manufactured by Perkin Elmer (Waltham, MA). Mice were removed from the study and euthanized when ill- appearing or the luciferase signal exceeded 10^8 photons/second, whichever came first. Anti-CD8 (clone 2.43), anti-CD4 (clone GK1.5), anti-NK1.1(PK136) and anti-CSF1R(AFS98) were purchased from Bio X Cell (West Lebanon, NH). Recombinant murine IL-12-p70 was purchased from Peprotech (Rocky Hill, NJ).

Ex vivo NK cytotoxicity assay

Leukemia cells were cultured in RPMI medium + 10% FBS + 1% penicillin/streptomycin + 0.1% 2-ME in a 37°C incubator. Cells were plated at 0.5–2 × 10^5 cells/ml and split every 48–72 hours. For the cytotoxicity assay experiments, murine NK cells were obtained from splenocytes of C57BL/6 mice using EasySep™ Mouse NK Cell Isolation kit (STEMCELL Technologies) were isolated. Either shNS or shCnB leukemia cell stained with Cell Tracker Green (CTG) and then plated at varying E:T ratios Percent CTG^+ cells were assessed via flow cytometry for NK cytolytic killing.

Statistics

Statistical analyses were performed using GraphPad Prism software. Statistical significance between 2 groups was determined by Student's t test, while Analysis of Variance (ANOVA) with Tukey's multiple comparison test was used to test significance

between 3 or more groups. Error bars in figures represent the standard deviation and may be obscured when narrow. Animal experiments included at least 3 mice/group, and all mice are included in survival analyses. To minimize animal use, *in vivo* experiments were repeated only once, unless results were inconclusive, in which case the experiment was repeated a third time. The Mantel-Cox (log-rank) test was used to test for significant differences in survival.

3.4 Results

Natural killer cells are not critical to calcineurin-deficient leukemia clearance

We observed prolonged survival in rIL12-treated *Rag1*$^{-/-}$ or Tcra$^{-/-}$ shCnB mice with leukemia, suggesting the importance of components of the innate immune system in immune surveillance during leukemia progression. To identify the innate immune cell subsets responsible for the initial clearance of IL-12 secreting leukemia, we hypothesized that NK cells are involved in the protection seen in *Rag1*$^{-/-}$ mice as IL-12 enhances the cytotoxicity of NK cells [168]. This hypothesis is supported by murine models that have shown that dendritic cell production of IL-12 is necessary to control metastasis in which the main effector cells are NK cells [169], and that exogenous IL-12 is able to rescue the cytotoxicity of NK cells previously rendered anergic by the tumor [169]. We evaluated the role of NK cells in calcineurin-dependent immune evasion. *In vivo* depletion of NK cells with anti-NK1.1 antibody did not affect survival outcomes in immune-competent mice (**Fig 3.4A**). Further, examining cytolytic activity of NK cells in eliminating calcineurin-deficient leukemia exhibited no difference in killing regardless of the effector to target

(E:T) ratio (**Fig. 3.4B**). We concluded NK cells are not responsible for the prolonged survival we observed in rIL-12 treated *Rag1*$^{-/-}$ mice.

Depletion of CSF1R^{+} immune cells are important for clearance of calcineurin-deficient leukemia

Depletion of NK cells with anti-NK1.1 did not abrogate the elimination of an immunogenic model of leukemia, in which calcineurin has been knocked down by shRNA. Thus, the role of myeloid cells in clearance of leukemia was examined. The depletion of myeloid cells from immunocompetent mice using colony-stimulating factor-1 receptor (CSF1R) depleting antibody promoted the progression of calcineurin-deficient leukemia (**Fig. 3.5A**). This was eliminated in all isotype-treated, immune-competent recipients, indicating myeloid cells play a critical role in the immune response to ALL cells. Due to the IFN-γ release in the B-ALL microenvironment due to IL-12 activation and expansion of cytotoxic CD8^{+} T-cells, this likely induces antigen presentation in the myeloid compartment as seen in previous study for B-ALL [170].

T-cells are responsible for leukemia clearance in leukemia-cell calcineurin model

The clearance of leukemia was observed in wild-type mice with an intact adaptive immune system, but not in Tcra$^{-/-}$ mice deficient in mature T-cells. To examine if either CD4^{+} or CD8^{+} T cell subsets are sufficient for immune surveillance and suppression of calcineurin deficient leukemia cells, we used monoclonal antibodies to selectively deplete these populations in WT recipients prior to challenging them with leukemia. Consistent

with a critical role of both $CD4^+$ and $CD8^+$ cells in adaptive immune responses, depletion of either population resulted in progression of calcineurin-deficient leukemia at rates similar to depletion of both and similar to the rate in $Tcra^{-/-}$ recipients (**Fig. 3.6A**). Overall, this data shows that CnB-deficient leukemia cells secrete pro-inflammatory molecules, IL-12 particularly, which leads to the activation of $CD4^+$ and $CD8^+$ T-cells to promote an effective immune response against leukemia, which may have clinical relevance in children with ALL.

Discussion

Although leukemia remains a leading cause of death in children, harnessing the potency of the immune system is emerging as a viable strategy for improving outcomes. In our previous work, we discovered a calcineurin-dependent immune evasion mechanism in an ALL model. ALL calcineurin-deficient cells can expand for several days in immune-competent mice, but they are eventually repressed to the point of becoming undetectable, and around half of the recipients never relapse. We also discovered calcineurin-deficient leukemia cells release more IL-12, which is responsible for T cell activation differences *in vitro,* resulting in robust IFNγ production consistent with a Th1 response. Treatment with rIL-12 improved long-term survival in immune-competent mice with ALL, confirming its previously documented function in antitumor immunity.

While not fully understood, there is a growing body of knowledge about immune evasion mechanisms during leukemogenesis [66], not all of which are shared with solid malignancies. However, to our knowledge, this is the first report of an intracellular signaling molecule in leukemia cells playing a significant role in immune evasion during leukemia progression. These findings are similar to the discovery that melanoma-

intrinsic-catenin signaling promotes immune evasion [171]. Melanoma with active-catenin fail to recruit T-cells to the tumor microenvironment, at least in part, due to reduced CCL4 secretion. Similarly, leukemia cells with active calcineurin fail to trigger an adaptive immune response in our model, at least in part due to reduced IL-12 secretion. These findings highlight an understudied aspect of immune evasion, the repression of danger signals from cancer cells that would otherwise elicit an effective immune response.

Our data support the development of strategies to promote IL-12 induced priming of T-cells resulting in the differentiation and activation of T-cells. IL-12 functions as a third signal, along with APCs and costimulatory signals to promote activation of naïve $CD8^+$ T-cells [172, 173]. In addition, IL-12 enhances the differentiation of effector and memory T-cells, at least in part, through regulation of T-box transcription factors T-bet and Eomes [174]. IL-12 induced effector $CD8^+$ T-cells by calcineurin-deficient leukemia cells, as evidenced by increased production of IFNγ and TNFα induced by the shCnB leukemia. Our data are similar to observations by other groups in models of melanoma and breast cancer, in which IL-12 was required for the generation of IFNγ-producing effector $CD8^+$ T-cells and antitumoral responses [175]. More broadly, these data are a reminder that enhancing T-cell immune responses may be considered a successful strategy to overcome immune suppression in B-ALL.

Notably, we also observed prolonged survival in rIL12-treated *Rag1*$^{-/-}$ mice with leukemia, demonstrating the contributions of innate immune cells to leukemia surveillance; however, further experimentation demonstrated that NK cells did not participate in anti-leukemia immunity (Fig. 3.4A). In contrast, $CSF1R^+$ myeloid cells were shown to be necessary in controlling calcineurin-deficient leukemia, exhibiting the importance of these immune cells in leukemia clearance. We found that cytokine

signaling is an important modulator of immune responses against ALL, as higher expression of IL15Rα by lymphoblasts was associated with better survival in children with relapsed ALL [176, 177]. Thus, whether higher expression of proinflammatory genes in ALL can be translated into a biomarker of protective inflammation and treatment outcome remains to be determined.

The clinical efficacy of IL-12 at tolerated doses has generally been limited, with the exceptions of cutaneous T-cell lymphoma, mycosis fungoides, and non-Hodgkin lymphoma [178–180]. Systemic administration is associated with toxicity, precluding dose escalation, and prompting the study of local delivery of IL-12 via gene therapy for solid tumors and leukemia [181], including in an ongoing clinical trial (NCT 02483312). Other methods of targeted delivery (i.e., oncolytic adenovirus and nanoparticles) have been tested in preclinical models demonstrating high antitumor efficacy with significantly lower systemic reactions [182, 183], and considering the dramatic success of CAR-T-cell therapy for ALL [184 – 186], we are now developing strategies for local delivery of IL-12 to activate T-cells and the role of IL-12 in overcoming the immune suppressive BM microenvironment. Further studies are currently being conducted in collaboration with our lab and a biomedical engineering lab to develop nanoparticle that will locally deliver IL-12.

In conclusion, we have found that IL-12 secreted by leukemia-cell calcineurin promotes an effective immune response in immune-competent mice during leukemogenesis. We identified both the importance of APCs and cytolytic T-cells in leukemia clearance. This data suggests modulating the leukemia microenvironment utilizing IL-12 may be an effective strategy to further improve leukemia therapy and has informed our next steps for an IL-12 therapeutic in ALL.

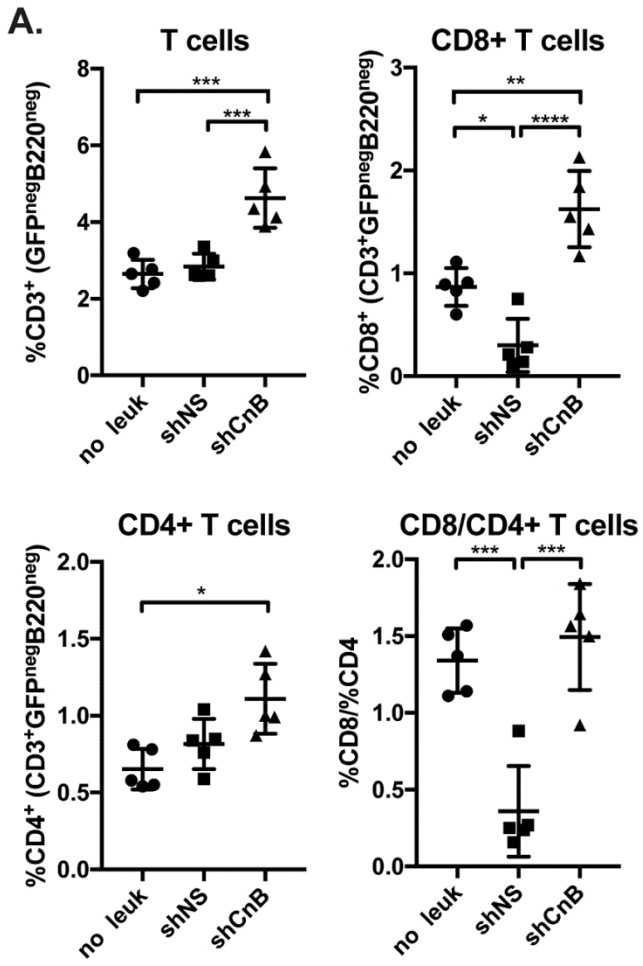


Figure 3.1 Depletion of leukemia-cell calcineurin elicits an adaptive immune response to leukemia cells. A. Bone marrow was harvested and analyzed by flow cytometry 7 days after injection of GFP^+ B ALL cells into un-irradiated WT recipient mice as in Figure 1. Data are representative of 2 independent experiments with 5 mice/group. (ANOVA with Tukey's multiple comparison test).

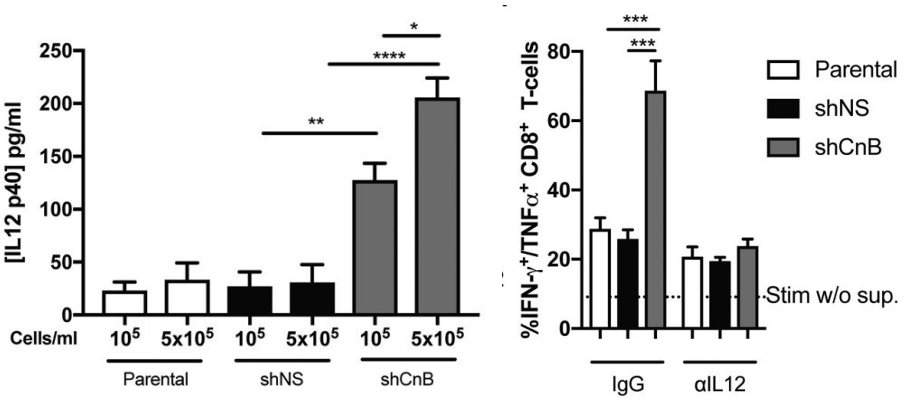


Figure 3.2. Calcineurin-deficient leukemia cells secrete IL-12 and activate T-cells. A. ELISA was used to measure IL12-p40 levels in the supernatant of parental, shNS and shCnB leukemia cells cultured *ex vivo*. (n=3 independent experiments; *P<0.05, **P<0.01, ****P<0.0001, ANOVA with Tukey's multiple comparisons test.) B. Murine splenocytes were stimulated *ex vivo* with CD3/CD28 antibodies and cultured with supernatant from shNS or shCnB leukemia cells with or without anti-IL12 neutralizing antibodies and analyzed by flow cytometry. The percentage of double positive $CD8^+$ cells.

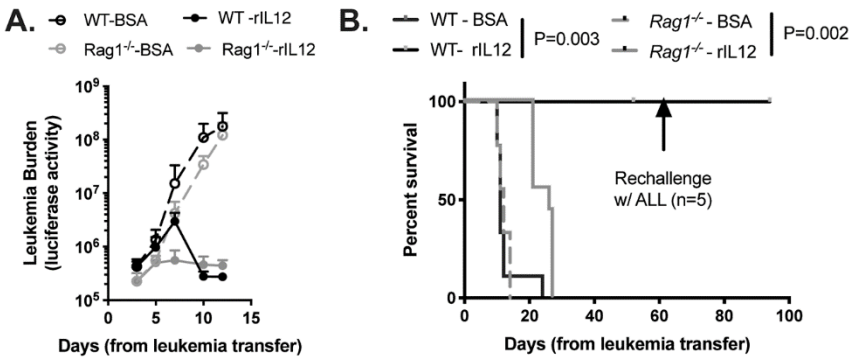


Figure 3.3. Recombinant IL-12 prolongs survival of both immune competent and immune deficient mice with leukemia. A, B. Parental BCR-ABL1^{+}/Arf$^{-/-}$ leukemia cells were injected into un-irradiated WT or Rag1$^{-/-}$ recipients. On day 3, treatment with either rIL12 or BSA was begun (1μg/dose, intraperitoneal, on days 3–7, 10–14 and 17; n=9/group from 2 independent experiments). Leukemia burden (**A**) and survival (**B**) were measured over time.

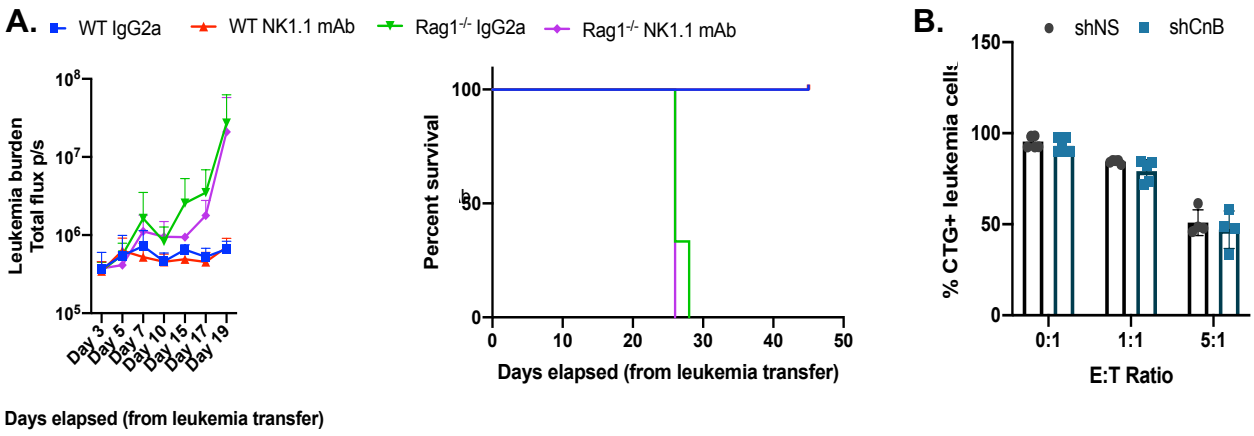


Figure 3.4. Natural killer cells are not critical to calcineurin-deficient leukemia clearance. A, B. Calcineurin-deficient leukemia BCR-ABL1^{+}/Arf$^{-/-}$ leukemia cells were injected into un-irradiated WT or Rag1$^{-/-}$ recipients. Mice were treated with either isotype control or NK1.1 mAb every two days post-day 3 engraftment (500μg/dose, intraperitoneal; n=9/group from 2 independent experiments). Leukemia burden (**A – left panel**) and survival (**A – right panel**) were measured over time. (**B**) Natural killer cells were isolated and co-cultured at varying ratios with either shNS or shCnB leukemia cell stained with Cell Tracker Green (CTG). Percent CTG^{+} cells were assessed via flow cytometry.

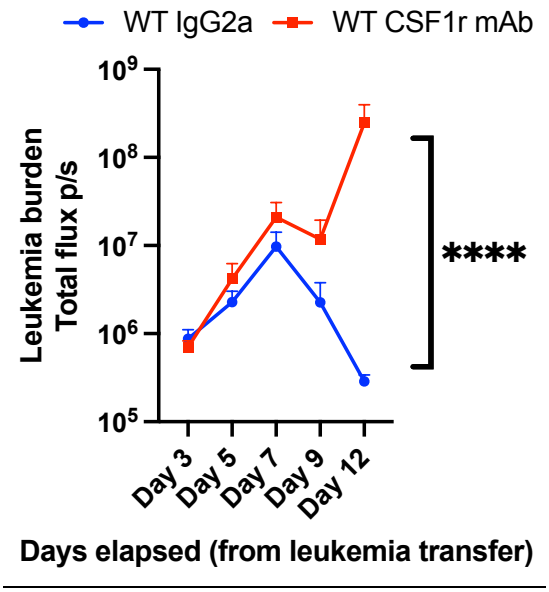


Figure 3.5. Depletion of CSF1R$^+$ immune cells are important for clearance of calcineurin-deficient leukemia. BCR-ABL1$^+$/Arf$^{-/-}$ leukemia cells were injected into un-irradiated WT recipients. Mice were treated with either isotype control or CSF1R mAb every two days post-day 3 engraftment (500μg/dose, intraperitoneal; n=9/group from 2 independent experiments). Leukemia burden was measured over time.

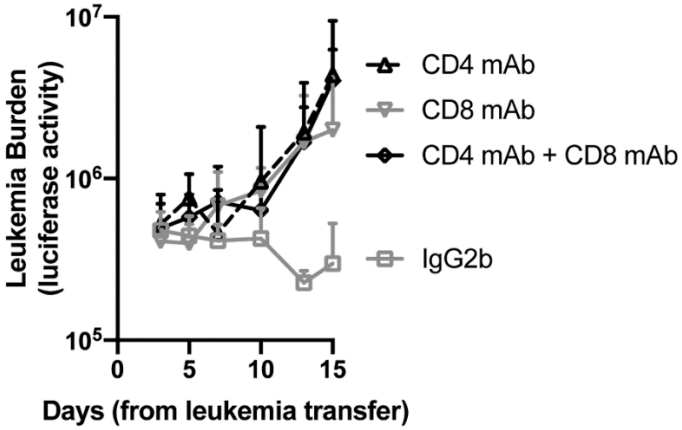


Figure 3.6. T-cells are responsible for leukemia clearance in leukemia-cell calcineurin model. WT mice were injected with neutralizing antibodies directed against CD4 and/or CD8 followed by injection of shCnB ALL. (n=6/group from 2 independent experiments)

Chapter 4

B-cell acute lymphoblastic leukemia promotes an immune suppressive microenvironment that can be overcome by IL-12

This chapter is adapted from a manuscript submitted by Hunter et al. *B-cell acute lymphoblastic leukemia promotes an immune suppressive environment*. In press. *Scientific Reports*, 2022.

4.1 Abstract

Immunotherapies have revolutionized treatment of B-cell acute lymphoblastic leukemia (B-ALL), but the duration of responses is still sub-par. Thus, we sought identify mechanisms of immune suppression in B-ALL and therapeutic strategies to overcome them. Plasma that was collected from children with B-ALL with measurable residual disease after induction chemotherapy exhibited differential cytokine expression, particularly IL-7. Single cell RNA-sequencing revealed the expression of genes associated with immune exhaustion in immune cell subsets. We also discovered that the supernatant of leukemia cells suppressed T-cell function *ex vivo*. Modeling B-ALL in mice, we observed an altered tumor immune microenvironment, including compromised activation of T-cells and dendritic cells (DC). However, recombinant IL-12 (rIL-12) treatment of mice with B-ALL restored the levels of several pro-inflammatory cytokines and chemokines in the bone marrow and increased the number of splenic and bone marrow resident T-cells and DCs. RNA-sequencing of T-cells isolated from vehicle and rIL-12 treated mice with B-ALL revealed that the upregulation of genes associated with exhaustion, including *Lag3*, *Tigit,* and *Pdcd1,* was abrogated with IL-12 treatment, relative to vehicle-treated mice. In addition, the cytolytic capacity of T-cells co-cultured with B-ALL cells was enhanced when IL-12 and blinatumomab treatments were combined. Overall, these results demonstrate that the leukemia immune suppressive microenvironment can be restored with rIL-12 treatment which has direct therapeutic implications.

4.2 Introduction

Acute lymphoblastic leukemia (ALL) is the most common cancer in children and remains a leading cause of illness related death [187], primarily related to relapsed

disease. Current treatment strategies include highly toxic chemotherapy delivered at the cusp of tolerability [188]. Engaging the immune system using chimeric antigen receptor (CAR) expressing T-cells or the bispecific antibody blinatumomab, is highly effective at inducing responses and has revolutionized treatment of relapsed disease. But both strategies have considerable limitations, most importantly sub-optimal duration of response [189, 190], highlighting the limitations of current strategies.

Further, the development and progression of leukemia is, in part, due to the ability of the leukemia cells to evade immune elimination. Although some mechanisms of immune evasion are similar in both hematological and solid cancers, leukemia and lymphoma have unique methods of evading the immune system [68, 191 - 194]. Leukemia is known to alter the cellular and soluble composition of the BM [195 – 198]. Lack of antigen presentation and processing have been shown to contribute to the regulation of immune-cell tolerance in hematological malignancies [68, 191]. Further, overexpression of PD-L1 co-inhibitory ligand on leukemia cells and changes in both immunostimulatory and immune suppressive cytokines have been shown to inhibit cytotoxic T-cells that are significant for clearance of leukemia [68, 191]. Nonetheless, how leukemia cells evade the immune system remains incompletely understood.

Thus, we sought to better understand the molecular and cellular mechanisms of leukemia cell mediated immune evasion. We first defined the tumor immune microenvironment in children with B-ALL by measuring circulating cytokine levels at the time of diagnosis, as well as single-cell RNA sequencing of non-leukemia cells. We observed differential cytokine expression in those with and without measurable residual disease (MRD) after induction chemotherapy and a gene expression profile consistent with immune exhaustion in those with MRD. Modelling T-cell function *ex vivo*, we

observed a reduction in T-cell activation markers, CD44 and CD107a, on effector T-cells cultured in supernatant from human and murine B-ALL cell lines. We found further evidence of leukemia-induced immune suppression in a murine model of B-ALL that closely resembles human disease [164, 199]. Treating leukemia-bearing mice with rIL-12 restored T-cell numbers in the BM, consistent with our previous findings [199]. Furthermore, the number and activation state of dendritic cells (DCs) was also increased with rIL-12 treatment. In addition, rIL-12 treatment also altered levels of immunostimulatory cytokines and chemokines in the bone marrow. With targeted RNA-sequencing, we found upregulation of immune exhaustion genes in T-cells, including *Lag3*, *Tigit*, and *Il10,* in mice with leukemia compared to those without. Further, *H2-Eb1* and *H2-Ab1* were highly upregulated in IL-12 treated mice compared to mice without leukemia, indicating genes associated with activated T-cells acquiring major histocompatibility complex molecules related to memory T-cell homeostasis. Lastly, to determine the clinical implications of our findings, we determined if rIL-12 treatment impacted the efficacy of the immunotherapy, blinatumomab. In these studies, we found that B-ALL conditioned media attenuated the cytolytic capacity of T-cells when co-cultured with leukemia cells in the presence of blinatumomab, whereas treatment with rIL12 augmented the efficacy of blinatumomab in this condition. Together, these data provide mechanistic insight into B-ALL induced immunosuppression and highlight the therapeutic potential of IL-12 as a novel treatment for this disease.

4.3 Materials and Methods

Single Cell RNA-sequencing

Peripheral blood or bone marrow from de-identified children with B-ALL was collected at the time of diagnosis after informed consent for biobanking. All experiments

using human samples were approved by the Emory University Institutional Review Board (IRB# 00034535 and 00089506). Frozen, Ficoll-separated samples from 4 children with MRD and 3 without MRD were available for analysis. Samples were sorted by flow cytometry for $CD19^{+}CD10^{+}$ lymphoblasts or $CD45^{+}CD19^{neg}CD10^{neg}$ non-leukemia cells. The transcript data was processed using the Seurat package in R® statistical software. Quality control was implemented on the samples to retain cells only with transcripts for more than 200 unique genes and less than 30% mitochondrial contribution. All samples, both leukemic and nonleukemic, were then combined into one data object to ensure that flow cytometry had effectively separated malignant cells from peripheral immune cells. The combined data object was log-normalized and scaled using the most highly variable genes in the dataset. Principal component analysis (PCA) was performed, and Shared Nearest Neighbor (SNN) analysis was implemented in Seurat's FindNeighbors function, followed by a Uniform Manifold Approximation and Projection (UMAP) dimensional reduction technique, both using a dimensions parameter of 1:35. Finally, a UMAP plot was constructed, and clusters were visually separated. The groups of putative non-leukemia cells that mapped with the leukemia clusters were relabeled to reflect their true malignant identities. The few leukemia cells that mapped to the immune cell regions were omitted from downstream analyses. Once the cells were labeled to reflect their true malignancy states, analyses of non-leukemia and leukemia cell populations were performed separately. A total of 18,974 single cells were retained for further analysis: 5,906 nonleukemic immune cells and 13,068 leukemic cells (10X Genomics). Two methods were used to assign each cell a score based on their expression of exhaustion-specific marker genes. The first was calculated by totaling the normalized expression values for each of the exhaustion genes for each cell. The second resulted from the

summed raw number of exhaustion gene transcripts per cell, normalized by the total transcripts per cell. In each case, the distribution of scores across all cells was assessed, and cells having upper-outlier score values in the two distributions were noted and labeled as highly exhausted cells. Genes included in the exhaustion score were *PDCD1, CTLA4, HAVCR2, TIGIT, TOX, LAG3, NFATC1, NFATC2, NR4A1, TOX2, TCF7, CD244, CD160,* and *ICOS* [20].

Mice

C57BL/6, and $Rag1^{-/-}$ (B6.129S7-Rag1tm1Mom/J) mice were housed in microisolators in standard conditions at Emory University (Atlanta, GA). All experimental protocols and methods reported here have been carried out in accordance with rules on animal welfare and regulations under the ethical approval by Emory University Institutional Animal Care and Use Committee (IACUC). Animal studies were designed, executed, and reported consistent with ARRIVE guidelines.

Leukemia Model

The luciferase-expressing, BCR-ABL1 $Arf^{-/-}$ B-ALL cell line was originally provided by Dr. Richard Williams (St. Jude Children's Research Hospital, Memphis, TN) [166, 167]. Cells were transduced with MSCV-iresGFP and sorted for GFP expression for some experiments. For *in vivo* experiments, a total of 2×10^5 cells were transferred via tail vein injection into unirradiated, 6- to 8-week-old, female, wild-type (WT) C57BL/6 mice, with 3 mice per group (mice were randomly allocated to groups of mice without leukemia, vehicle treated mice with leukemia and rIL12 treated mice with leukemia). After intraperitoneal injection of luciferin and anesthesia with inhaled isoflurane, leukemia burden was measured by the In Vivo Imaging System (IVIS; Perkin Elmer). Mice were removed from the study and euthanized (per IACUC approved procedures) when ill-

appearing or the luciferase signal exceeded 10^8 photons/second, unless otherwise specified. Recombinant murine IL-12p70 was purchased from PeproTech and administered, unblinded, by intraperitoneal injection (1μg/dose) daily for 5 days, beginning 3 days after leukemia transfer.

***Ex vivo* Leukemia Cell Culture**

REH, RCH-ACV1 and NALM6 cell lines were acquired from ATCC. Leukemia cell lines were cultured in RPMI medium + 10% FBS + 1% penicillin/streptomycin + 0.1% 2-ME in a 37°C incubator. Cells were plated at 0.5–2 × 10^5 cells/ml and split every 48–72 hours. For B-ALL supernatant collection, leukemia cells were plated at 5×10^5 cells/ml and cultured for 48 hours before collection of supernatant.

T-Cell Activation and Cytotoxicity Assay

Human PBMCs were isolated from de-identified, normal donor buffy coats purchased from ZenBio (Durham, NC) or the Clinical and Translational Discovery Core (CTDC) at Children's Healthcare of Atlanta using a Ficoll-density gradient. $CD3^+$ T-cells were positively selected from PBMCs via magnetic separation (Miltenyi Biotech, CD3 Microbeads, Cat#130-050-101). T-cell purity was assessed at > 90% $CD3^+$ of live cells, and the cells were cryopreserved. Cells were cultured in RPMI (10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin) at 37°C, 5% CO2 unless otherwise specified. For T cell activation experiments, $CD3^+$ T-cells were stained with tracking dye CellTrace Yellow (ThermoFisher) prior to culture. After rest, T- cells were then plated in a 96-well u-bottom plate at 3.5 x 10^5 cells/well. Plated cells were then stimulated with either stimulation cocktail (eBioscience) or Dynabeads™ Human T-Activator CD3/CD28 (ThermoFisher). On day 2 of culture, cells were harvested, and magnetic beads removed. Cells were then stained with CD44 APC (IM7, eBioscience), CD107a Pe-Cy5 (H4A3, BioLegend), and Live

Dead Violet (Invitrogen) for 15 minutes in 4° in the dark. Cells were then washed twice with FACS buffer. For cytotoxicity experiments, Nalm-6 and $CD3^+$ T-cells were stained with tracking dyes CellTrace Violet (Thermo) and CellTrace Yellow (Thermo), respectively, prior to culture. Nalm6 cells were then plated in a 96-well u-bottom plate at a 1:1 E:T ratio in RPMI-1640 (10% FBS) or Nalm6 supernatant and treated with vehicle (PBS) or 0.5ng/mL-1 ng/mL blinatumomab (Invivogen, bimab-hcd19cd3) and/or 5-20 ng/mL rIL-12p70 (PeproTech) for 72 hours. The cells were then prepared for flow cytometry. Live/Dead APC dye (Invitrogen) used to identify dead Nalm-6 cells.

Murine T-cells were obtained from splenocytes of C57BL/6 mice using EasySep™ Mouse T Cell Isolation kit (STEMCELL Technologies) or CD4/CD8 Microbeads (Miltenyi Biotech). $CD3^+$ T-cells were stained with tracking dye CellTrace Yellow (ThermoFisher) prior to culture. After rest, T-cells were then plated in a 96-well u-bottom plate at 1 x 10^6 cells/well. Plated cells were then stimulated with either stimulation cocktail (eBioscience) or murine Dynabeads™ Mouse T-Activator CD3/CD28 (ThermoFisher). On day 2 of culture, cells were harvested, the magnetic beads were removed, and cells were stained for flow cytometry.

Flow Cytometry

Samples were collected on the Cytek Aurora or Cytoflex flow cytometer (Beckman Coulter) and analyzed using FlowJo software (Tree Star). Murine bone marrow samples from *in vivo* and *in vitro* experiments were acquired and stained using the following antibodies: anti–mouse CD16/32 (2.4G2; Fc block) from BD Biosciences; anti-mouse CD107b Alexa Fluor® 647 (M3/84), CD8a BV510 (SK1), CD3ε Alexa Fluor® 700 (500A2), CD11c APC (N418), CD4 APC/Fire™ 810 (GK1.5), CD127 Brilliant Violet 421™ (A7R34), CD80 Brilliant Violet 510™ (16-10A1), Ly-6C Brilliant Violet 570™ (HK1.4),

CD86 Brilliant Violet 605™ (GL-1), Brilliant CD107a Violet 711™ (1D4B), CD11b Brilliant Violet 750™(M1/70), CD62L Brilliant Violet 785™ MEL-12), CD45R/B220 Pacific Blue™ (RA3-6B2), CD279 PE anti-mouse (29F.1A12), CD274 PE/Cyanine7(10F.9G2), Ly-6G PE/Dazzle™ 594 (1A8), KLRG1 PE/Dazzle™ 594 (2F1/KLRG1), CD44 PerCP/Cyanine5.5 (IM7), CD107b BV650 (CD8a Spark Blue™ 550 (53-6.7), Zombie NIR™ Fixable Viability Kit, all from BioLegend; anti–mouse MHC Class II (I-A/I-E) PerCP-eFluor 710 (M5/114.15.2), CD44 APC (IM7) from eBioscience; CD107b APC-Vio770 (M3/84) from Miltenyi Biotec. Primary human T-cells were stained for purity post-isolation using anti-CD3 (OKT3, BioLegend), and Near IR Live/dead stain (Invitrogen).

Cytokine ELISAs and Luminex Assays

Plasma from children with B-ALL was analyzed for cytokine and chemokine concentrations using the Cytokine 35-Plex Human Panel (ThermoFisher) for the Luminex platform. Supernatant acquired from T-cells cultured *in vitro* at a cell density of 1×10^6 cells/well (murine) or 3.5×10^5 cells/well (human) were collected after 48 hours. The concentration of interferon-gamma (IFN- γ) in the supernatants was determined using either the mouse or human IFN-γ ELISA kit (RayBiotech; cat no. ELH-IFNg-1) per the manufacturer's instructions. Absorbance at 450 nm was recorded using the Synergy 2 multi-mode microplate reader (Biotek). Murine bone marrow supernatant was harvested on day 7 post-engraftment from WT mice treated with rIL-12p70 or untreated for 5 days. Bone marrow serum was acquired in 0.5% BSA in PBS at 500 µl was analyzed by Eve Technologies (Calgary, AB, Canada) utilizing mouse cytokine and chemokine array 44-plex at 1-fold dilution.

Quantitative Gene Expression Analysis

Mouse bone marrow and spleen was harvested seven days after transplantation of luciferase–expressing BCR-ABL1^{+} B-ALL cells transduced with GFP. Pan-T cell kit (Miltenyi Biotec) was used to isolate murine T-cells from the bone marrow of mice. RNA isolation and sequencing was performed by the Integrated Genomics Core at Emory University using nCounter® Immune Exhaustion Panel (nanoString, Seattle, WA) that profiles 785 genes across 47 pathways. Data was normalized using Nsolver database and log10 fold change was assessed for heat map generation. Protein-protein interaction (Ppi) differences were imported from STRING (https://string-db.org/) into Cytoscape using the list of Uniprot IDs (crosschecked with STRING as needed).

Statistical Analysis

Most statistical analyses were performed using GraphPad Prism software. Statistical significance between two groups was determined by a Student's t-test, while ANOVA with Tukey multiple comparison test was used to test significance between three or more groups. Error bars in figures represent the SD and may be obscured when narrow. Animal experiments included at least 3 mice/group, and data from all mice are included. To minimize animal use, *in vivo* experiments were repeated only once. A false discovery rate (FDR) with adjusted p-value of 0.05 was used to generate volcano plots. Gene ontology data was generated based on at least two-fold up- or down- regulation compared to the no leukemia control, and p-value < 0.05. Heatmaps and hierarchical clustering were generated using Morpheus (https://software. broadinstitute.org/morpheus). The datasets generated are available in the NCBI GEO data repository (GSE198519).

4.4 Results

Changes in the immune microenvironment of B-ALL at the time of diagnosis are associated with MRD

The immune microenvironment is both influenced by and influences the development of B ALL [192 – 196]. As MRD at the end of induction chemotherapy is the strongest predictor of relapse in children with B ALL [188], we sought to compare the immune microenvironment at the time of diagnosis in those with and without MRD. We first measured a panel of cytokines and chemokines in the plasma in children with B ALL. After correction for multiple comparisons, among the analytes measured and reliably detected, only IL-7 was detected at different levels, with higher levels in those without MRD (**Fig. 4.1A**). In addition, the ratio of IL-7 to both IL-1 and IL-1b was significantly higher in those without MRD (**Fig. 4.1B**).

To determine the impact of MRD on the cellular immune microenvironment, we focused analyses of single-cell RNA-sequencing (scRNA-seq) on non-malignant immune cells derived from pediatric patients with B-ALL. Cells were clustered based on the expression of genes associated with specific hematopoietic subsets (**Fig. 4.1C**). Among, the defined subsets, only the erythroid precursor population was significantly associated with outcome, with greater percentages of erythroid precursors in those without MRD (38.7% v. 7.3%, $P<0.0001$, 2-way ANOVA with Sidak's multiple comparison test). We next focused on genes involved in immune exhaustion and identified more cells with high immune exhaustion scores in those with MRD at the end of induction therapy, including T-cells and natural killer (NK) cells, which are important for eradicating leukemia cells (**Fig. 4.1D**). These results demonstrate that the presence of residual disease coincides

with immune dysfunction at the time of diagnosis, notably cytotoxic cells with exhausted gene expression signatures.

The B-ALL secretome suppresses T-cell activation

Based on these results, we next determined how B-ALL cells directly impact T-cell activation. Human $CD3^+$ T-cells were stimulated *in vitro* with either anti-CD3/CD28 or PMA/ION and cultured in unconditioned media or conditioned media derived from the supernatants of Nalm-6, REH, or RCH-AcV human B-ALL cell lines (**Fig. 4.2A**). Human T-cells stimulated in B-ALL supernatants expressed significantly lower levels of surface markers associated with T-cell activation including CD44 (which plays a role in T-cell adhesion [200] and CD107a (a lysosomal protein transported to the T-cell surface during the degranulation of cytolytic content (201, 202; **Fig. 4.2B**). Similarly, the activation of murine T-cells was suppressed by the B-ALL secretome, resulting in lower surface expression of CD44 and CD107b expression (another surface marker of degranulation (203; **Fig. 4.2C**). In addition to the suppression of surface proteins associated with T-cell activation, the production of effector cytokines by human $CD3^+$ T-cells, notably IFN-γ, was significantly inhibited by B-ALL secreted factors (**Fig. 4.2D**). These data supported our scRNA-seq results demonstrating that T-cells suppression occurs in the B-ALL microenvironment and provided direct evidence that the B-ALL secretome potently suppresses T-cell effector function.

B-ALL induces cellular changes in the B-ALL microenvironment that can be normalized by IL-12

To model immune microenvironment changes *in vivo*, we used a well-characterized mouse leukemia, driven by BCR-ABL1, that rapidly engrafts and progresses lethally in non-irradiated, immune competent recipients [15, 16] With this model, we

previously demonstrated that IL-12 promotes T-cell dependent immune clearance of leukemia and prolongs survival [199]. Furthermore, we demonstrated that protection correlated with changes in T-cell subset numbers in the bone marrow [199]. When BM cells were harvested from mice 7 days after engraftment of leukemia cells, we found alterations in the number and activation state of several immune cell populations. Similar to previous observations [199], the most consistent leukemia-induced changes in the immune microenvironment were in the myeloid compartment (**Fig. 4.3A**). Specifically, we observed significant reductions in the total number of conventional ($CD11c^+CD11b^+$) and plasmacytoid ($CD11c^+B220^+$) dendritic cells. Treatment of the mice with leukemia using recombinant IL-12p70 (rIL-12) restored the numbers of these cells and enhanced the activation state of both conventional and plasmacytoid DCs, which expressed higher surface levels of T-cell costimulatory molecules (CD80 and CD86) and MHC class II molecules which present antigens to $CD4^+$ T-cells (**Fig. 4.3A**). In addition, mice with leukemia had changes in the T cell compartment, with higher proportions of $CD4^+$ cells and lower proportions of $CD8^+$ cells (**Fig. 4.3B**). However, treatment with rIL-12, increased the percentage of T-cells in the bone marrow, such that the number of T-cells was restored, as was the ratio of $CD8^+/CD4^+$ cells (**Fig. 4.3B**). As before [199], rIL-12 treatment of B-ALL-bearing mice significantly reduced leukemia burden at day 10 post-transplantation of leukemia cells. Together, these data demonstrate extensive remodeling of the cellular tumor immune microenvironment in response to leukemia, which can be normalized to some extent by treatment with rIL-12, perhaps by heightened T-cell priming capacities by resident DC (dendritic cells) subsets.

IL-12 treatment of B-ALL bearing mice creates an immunostimulatory soluble milieu in the bone marrow

We next sought to determine the extent to which leukemia alters immune signaling systems in the bone marrow, as well as changes induced by rIL-12. To this end, we performed a multiplexed cytokine/chemokine assay from supernatant collected from the bone marrow 7 days after engraftment of leukemia. After excluding analytes that were not reliably detected, scaling for batch effects, and correcting for multiple comparisons, a few notable patterns of differential cytokine levels were of interest (**Fig. 4.4A**). First, was a set of cytokines/chemokines that was altered based on the presence of leukemia (**Fig. 4.4B**). Second was a set of analytes with differential levels due to leukemia but normalized by IL-12 (**Fig. 4.4C**). The most dramatic differences were seen in proinflammatory cytokines induced by rIL-12, including IFN-γ (**Fig. 4.4D**), consistent with a robust immune response to the leukemia cells. These results demonstrate that rIL-12 treatment of leukemia-bearing mice may augment anti-leukemia immunity by promoting an immunostimulatory cytokine/chemokine milieu.

Genes associated with T-cell exhaustion are induced in B-ALL-bearing mice

As we previously demonstrated that T-cells are essential for effective immune clearance of leukemia cells [199], we sought to determine the molecular mechanisms of T cell failure and success by performing targeted gene expression profiles of T-cells from the leukemia immune microenvironment. T cell populations from vehicle treated mice with leukemia exhibited significant upregulation of genes associated with immune exhaustion, such as *Lag3, Tigit* and *Il10*, as well as *Ms4a2,* an immune suppressive gene expressed by T-regulatory cells [204] whereas these genes were not as highly expressed in T-cells from rIL-12 treated mice with leukemia (**Fig. 4.5A, B**). In fact, the vast majority

differentially expressed genes in T-cells isolated from B-ALL bearing mice treated with rIL-12 were expressed at lower levels, as compared to T-cells from mice without leukemia, including some also downregulated in the vehicle treated mice with leukemia, suggesting leukemia-mediated suppression (**Fig. 4.5A, B**).

We then examined protein-protein interaction (Ppi) networks after grouping genes under functional annotations for our RNA-seq data, to identify how protein interactions in T-cells may be regulated under the conditions tested. This also demonstrates a trend in upregulation of genes associated with immune exhaustion in T-cells from leukemia-bearing relative to naïve mice (**Fig. 4.5C**), but to a lesser extent in those treated with IL-12 (**Fig. 4.5D**). Together, these data demonstrate upregulation of several genes in T-cells, in response to leukemia, including those associated with immune exhaustion, which is abrogated by treatment with IL-12.

Combining blinatumomab and IL-12 therapies overcomes B-ALL suppression of T-cells

Considering the critical role of T-cells in leukemia clearance and the current use of bispecific T-cell engaging antibodies (BiTE) for patients with relapsed ALL, we next investigated the efficacy of blinatumomab in combination with IL-12. Blinatumomab is composed of two different single-chain variable (Fv) fragments with a glycine-serine linker [205]. It is directed against CD3e human T-cell antigen and pan-B cell marker CD19 (expressed on B-ALL cells). We hypothesized that combination treatment would enhance T-cell-mediated killing of leukemia cells. To this end, human $CD3^+$ T-cells were co-cultured with Nalm6 cells (a human B-ALL cell line) and treated with either blinatumomab alone or in combination with IL-12 for 72 hours (**Fig. 4.6A**) We observed an increase in leukemia cell death with blinatumomab in combination with rIL-12

treatment compared to blinatumomab alone (**Fig. 4.6B**). There was also increased secretion of IFN-γ from $CD3^+$ T-cells treated with both blinatumomab and rIL-12 (**Fig. 4.6C**).

Based on these observations, we determined if combining blinatumomab and rIL-12 treatments overcome B-ALL-mediated suppression of T-cells. Notably, we observed that B-ALL secreted factors compromised blinatumomab-mediated killing of B-ALL cells when co-cultured with $CD3^+$ human T-cells (**Fig. 4.7A**). Interestingly, rIL-12 treatment overcame this suppression and resulted in a two-fold increase in the cytotoxicity of B-ALL cells when co-cultured with human T-cells and blinatumomab in leukemia cell supernatant (**Fig. 4.7B**).

In all, these data suggest that B-ALL mediated immunosuppression can be overcome with rIL-12 treatment, and this strategy may represent a novel approach to augment the efficacy blinatumomab in MRD-positive patients with B-ALL.

4.5 Discussion

Leukemia is a leading cause of disease-related deaths in children, and immunotherapies have emerged as revolutionary treatments for patients with refractory or relapse disease. Despite our best clinical efforts, immunotherapies targeting CD19-expressing malignant B-cells, notably blinatumomab and chimeric antigen receptor (CAR) T-cells, have failed to elicit long-term protection in over 40% of pediatric patients receiving treatment [206, 207] which highlights the need for novel strategies to optimize the efficacy of these groundbreaking treatments.

In these studies, we present data demonstrating that the presence of MRD in patients diagnosed with B-ALL is associated with immune cell exhaustion. In our *in vitro* and murine models of B-ALL, we found that similar T-cell responses were directly

induced by B-ALL secreted factors and the leukemic microenvironment, respectively. Human and murine T-cells in both contexts exhibited attenuated T-cell effector responses (significantly lower CD44 surface levels and reduced IFN-γ production) and gene expression profiles indicative of T-cell exhaustion (*Il10, Lag3, Tigit*). T-cell exhaustion has been observed in murine models of ALL and pediatric cases of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) where high numbers of TIM-3$^+$ CD4$^+$ T-cells is correlated with a higher risk of relapse [208]. Notably, we demonstrated that treatment with rIL-12 overcomes B-ALL-induced immunosuppression, highlighted by the establishment of an immunostimulatory leukemia microenvironment in the bone marrow, characterized by high levels of IFN-γ, IL-2, and various chemokines, higher numbers activated DC subsets in the bone marrow, and elevated numbers of highly functional CD8$^+$ T-cells. Importantly, we provide data supporting the adjuvant potential of rIL-12 treatment as a novel approach to overcome B-ALL-mediated immunosuppression in the context of blinatumomab treatment, where we observed enhanced killing of malignant B-cells when co-cultured with human T-cells with the addition of rIL12. Taken together, these data demonstrate the pleiotropic immunostimulatory potential of rL-12 treatment as a mechanism to improve outcomes in settings of B-ALL.

IL-12 has shown remarkable anti-tumor efficacy in a wide range of malignancies in preclinical studies, yet most of these studies are in solid tumors [209 – 211]. To our knowledge, we are the first to report 1) T-cell suppression by B-ALL supernatant, and 2) the reversal of this suppression in bone marrow resident T-cells in leukemia bearing mice treated with rIL-12. Our findings support previous research on IL-12 as a co-stimulatory molecule that induces T_H1 differentiation and increases activation and cytotoxicity of T-

cells [142, 212]. It also supports published animal model and clinical studies demonstrating IL-12-induced upregulation of pro-inflammatory mediators including IFN-γ from T-cells and the upregulation of MHC I and II surface expression on antigen presenting cells (APCs) [213 – 215].

We also identified enhanced efficacy of blinatumomab with IL-12 treatment in T-cells exposed to B-ALL supernatant compared to blinatumomab treatment alone. Our results demonstrate the potential for IL-12 to be used as a therapeutic in B-ALL patients. However, despite demonstrating potential anti-tumor activity in preclinical studies, systemic administration of IL-12 was associated with severe adverse effects in clinical trials [216 – 218]. Our results suggest that the targeted delivery of IL-12 and blinatumomab may be an effective strategy to overcome T-cell exhaustion associated with MRD (**Fig. 1**). In further support of this concept, we previously demonstrated that bi-specific T cell engaging (αCD19:αCD3) nanoparticles formulated to also deliver IL-12 to the immune synapse (termed BiTEokines) provided superior killing of human B-ALL cells when co-cultured with human T-cells relative to single-agent treatment with blinatumomab or rIL-12 [219]. In collaborative studies, we are currently in the process of testing the efficacy of BiTEokines in murine models of B-ALL.

In conclusion, our work demonstrates the potent immuno-rejuvenating potential of rIL-12 treatment in the context of B-ALL and demonstrates its potential as an adjuvant to improve the efficacy of blinatumomab treatment in patients with relapsed or refractory disease.

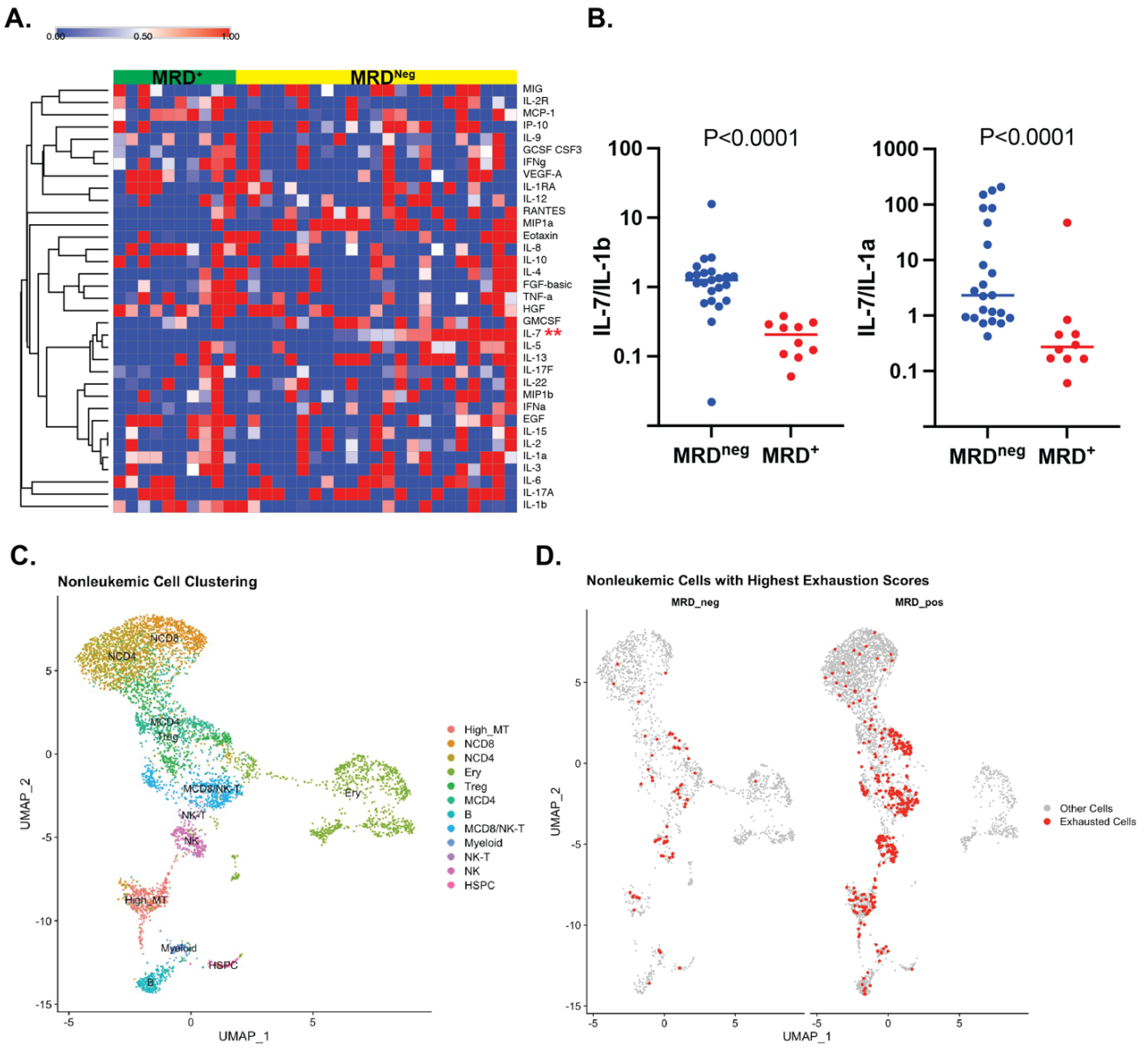
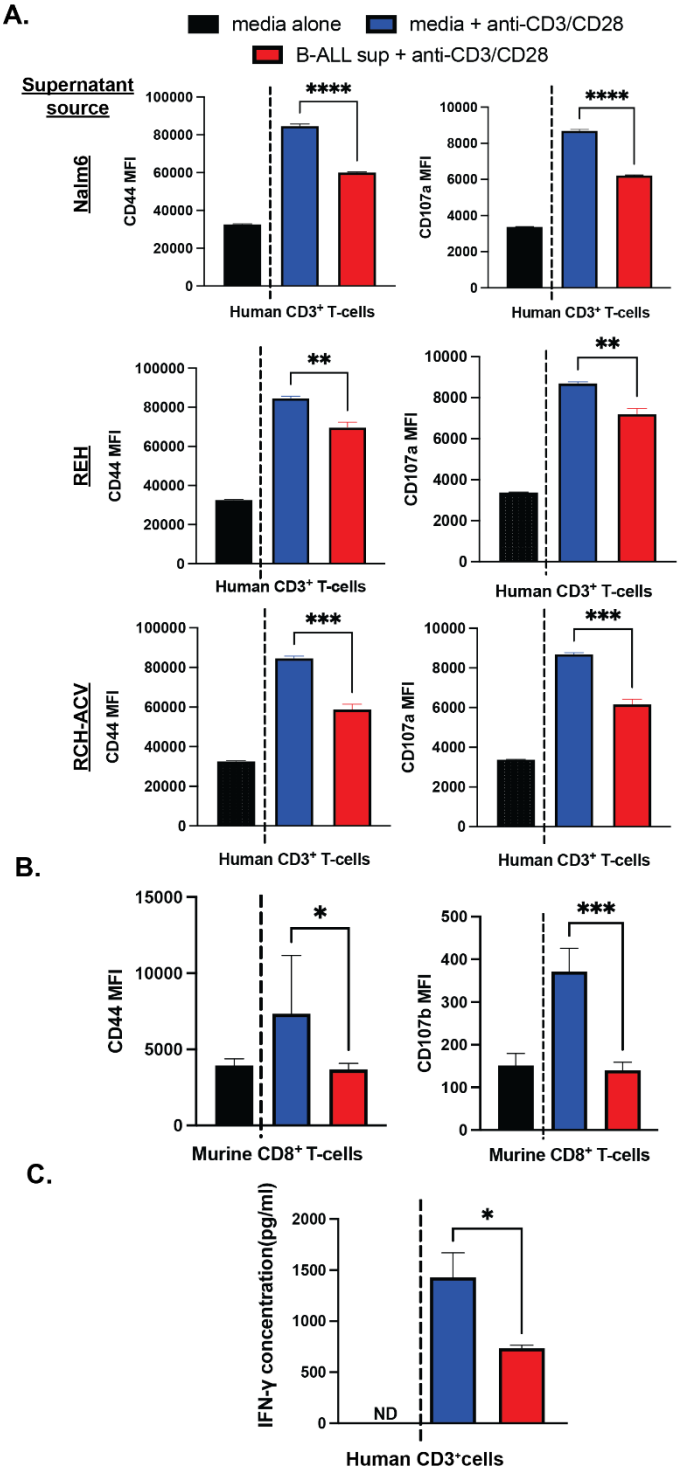

A.
0.00
0.50
1.00
MRD+
MRDNeg
MIG
IL-2R
MCP-1
IP-10
IL-9
GCSF CSF3
IFNg
VEGF-A
IL-1RA
IL-12
RANTES
MIP1a
Eotaxin
IL-8
IL-10
IL-4
FGF-basic
TNF-a
HGF
GMCSF
IL-7 **
IL-5
IL-13
IL-17F
IL-22
MIP1b
IFNa
EGF
IL-15
IL-2
IL-1a
IL-3
IL-6
IL-17A
IL-1b
B.
P<0.0001
IL-7/IL-1b
100
10
1
0.1
MRDneg
MRD+
P<0.0001
IL-7/IL-1a
1000
100
10
1
0.1
MRDneg
MRD+
C.
Nonleukemic Cell Clustering
NCD8
NCD4
MCD4
Treg
MCD8/NK-T
NK-T
NK
High_MT
Myeloid
HSPC
B
Ery
UMAP_2
UMAP_1
High_MT
NCD8
NCD4
Ery
Treg
MCD4
B
MCD8/NK-T
Myeloid
NK-T
NK
HSPC
D.
Nonleukemic Cells with Highest Exhaustion Scores
MRD_neg
MRD_pos
UMAP_2
UMAP_1
Other Cells
Exhausted Cells

Figure 4.1. B cell ALL alters the immune microenvironment. A. Cytokines were measured via Luminex from the plasma of peripheral blood of children with B ALL at the time of diagnosis. Cytokines are depicted in the heatmap relative to the median with supervised hierarchical clustering by residual disease at the end of induction chemotherapy (** $p<0.001$ t test; FDR q value = 0.002; two-stage step up (Benjamini, Krieger & Yekutieli)). **B.** The ratio of IL-7 to IL-1a and IL-1b is depicted (Mann Whitney test). **C.** Dimensional reduction of non-leukemia cell populations using UMAP with cells colored and labeled by immune cell assignment. **D.** Non-leukemia cells UMAP plot, split by disease outcome, with high exhaustion-scoring cells highlighted with high exhaustion scores in several cell populations including mature CD8 (MCD8) T-cells and natural killer T-cells (NK-T). Pearson's Chi-squared test with Yates' continuity correction used for statistical analysis demonstrates association of cells with high exhaustion score and MRD at end of induction chemotherapy (p-value < 5.2e-13).

Figure 4.2. The B-ALL secretome suppresses T-cell activation.

A. $CD3^+$ human T-cells stimulated *ex vivo* with CD3/CD28 antibodies were co-cultured in either Nalm6, REH or Rch-Acv supernatant for 48 hr and analyzed via flow cytometry. Graphs depict mean fluorescence intensity (MFI) for CD44 (left) and CD107a (right) antibodies (n = 2 independent experiments; *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$, ANOVA with Tukey multiple comparisons test) **B.** Murine splenocytes were stimulated *ex vivo* with CD3/CD28 antibodies and cultured with B-ALL supernatant (Nalm6) and analyzed by flow cytometry (n = 3 independent experiments; *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$, ANOVA with Tukey multiple comparisons test). **C.** ELISA for IFN-γ from supernatant of human T-cells cultured in B ALL conditioned media.



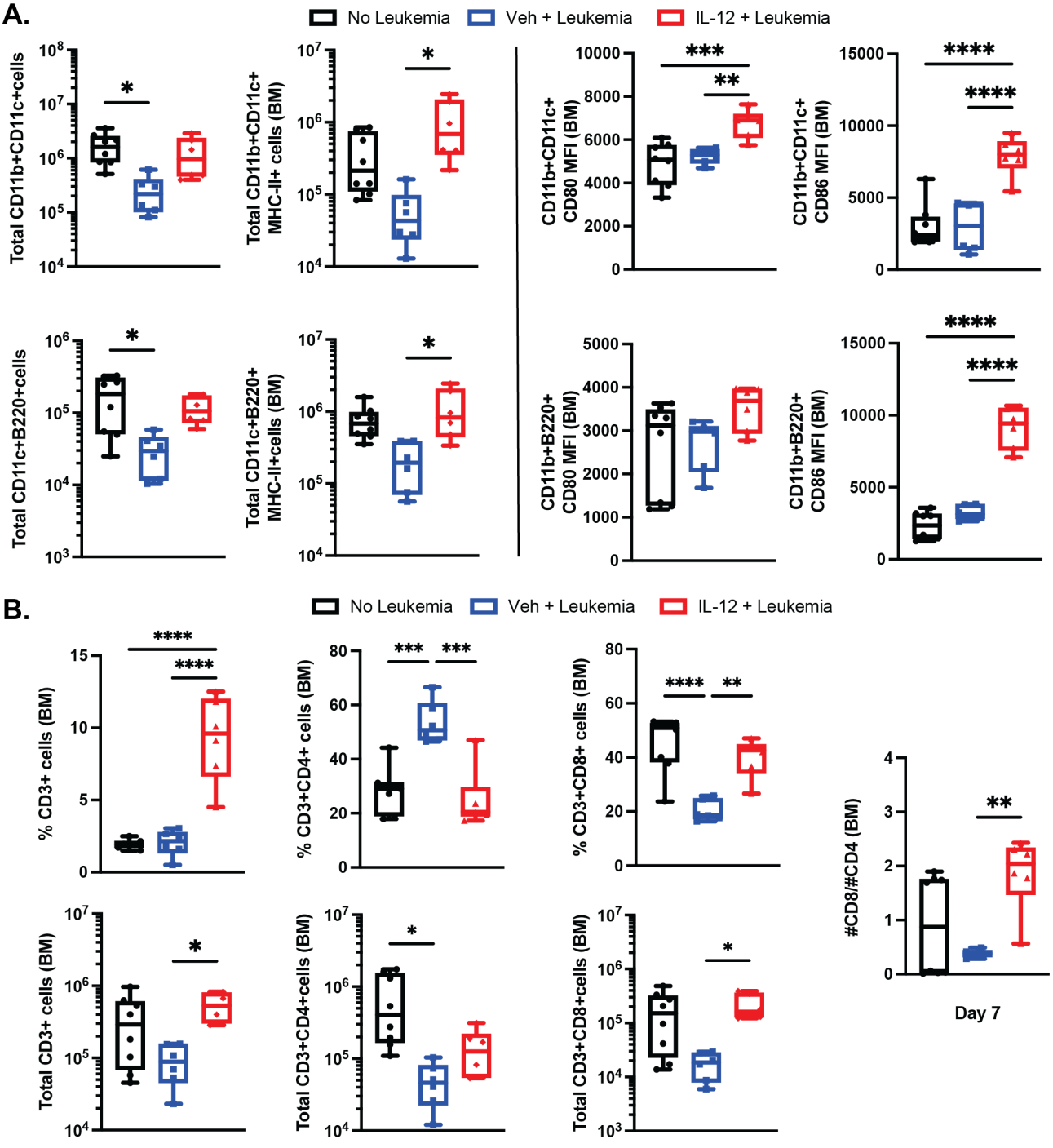

A.
No Leukemia
Veh + Leukemia
IL-12 + Leukemia
Total CD11b+CD11c+cells
Total CD11b+CD11c+ MHC-II+ cells (BM)
CD11b+CD11c+ CD80 MFI (BM)
CD11b+CD11c+ CD86 MFI (BM)
Total CD11c+B220+cells
Total CD11c+B220+ MHC-II+cells (BM)
CD11b+B220+ CD80 MFI (BM)
CD11b+B220+ CD86 MFI (BM)
B.
No Leukemia
Veh + Leukemia
IL-12 + Leukemia
% CD3+ cells (BM)
% CD3+CD4+ cells (BM)
% CD3+CD8+ cells (BM)
Total CD3+ cells (BM)
Total CD3+CD4+cells (BM)
Total CD3+CD8+cells (BM)
#CD8/#CD4 (BM)
Day 7

Figure 4.3. B-ALL induces cellular changes in the B-ALL microenvironment that can be normalized by IL-12. Parental BCR-ABL1^{+}/Arf$^{-/-}$ leukemia cells were injected into unirradiated WT recipients. On day 3, treatment with either rIL12 or BSA was begun (1 μg/dose, intraperitoneal, on days 3–7, 10–12); n = 6/group from two independent experiments). **A.** Total CD11b^{+}CD11c^{+} cells and CD11c^{+}B220^{+} cells in the bone marrow and CD86 and MHC-II mean fluorescence intensity (MFI) of each cell population, respectively. **B.** The percentage (top) and total number (bottom) of specified T cell populations in the bone marrow. The ratio of the number of CD8 to CD4 cells is also shown (right). (ANOVA with Tukey multiple comparison test).

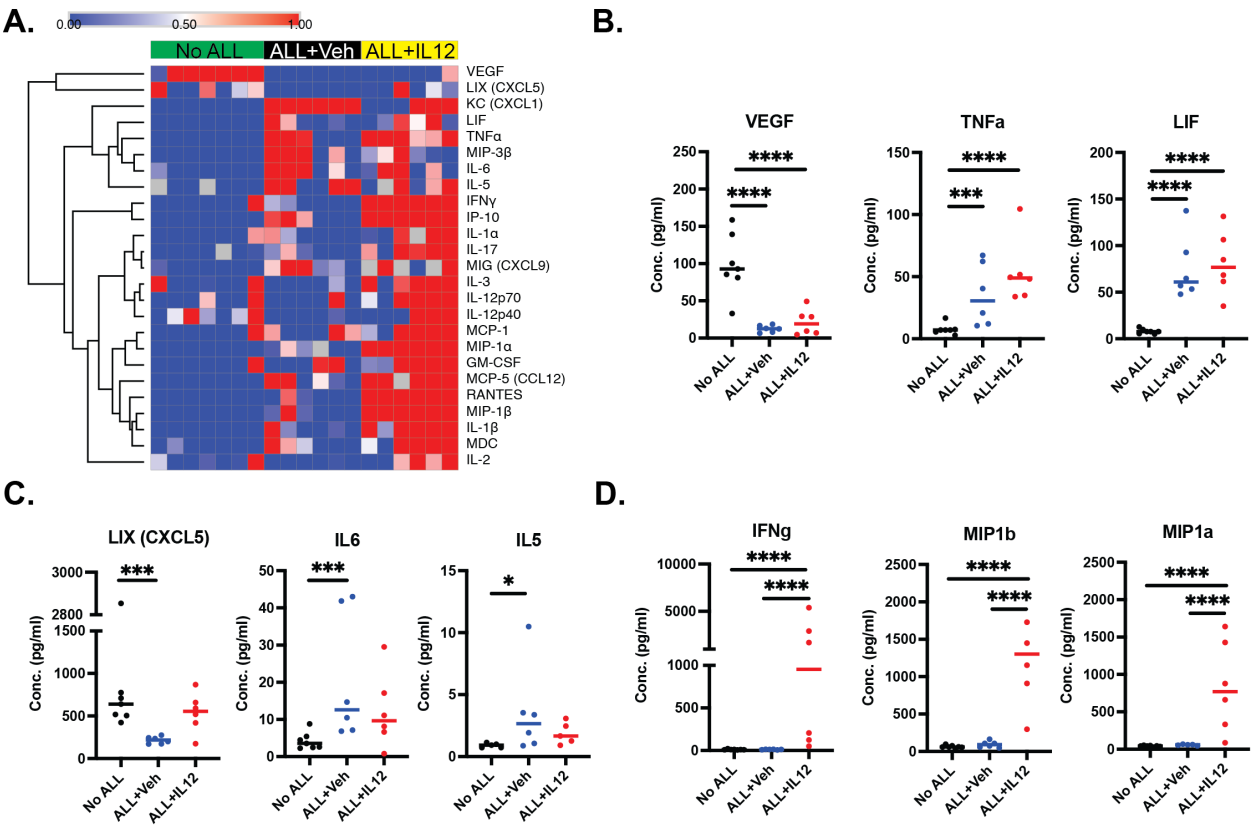


Figure 4.4. IL-12 treatment of B-ALL bearing mice creates an immunostimulatory soluble milieu in the bone marrow. A. Luminex assay was used to measure cytokines and chemokines levels in the bone marrow of control and rIL-12 treated WT mice at day 7 (n=6 mice/group). The heatmap shows concentrations relative to the median with supervised hierarchical clustering by treatment condition. **B.** Cytokines with altered concentrations attributed to leukemia. **C.** Cytokines/chemokines with altered concentrations due to leukemia and normalized with IL-12. **D.** Cytokines/chemokines altered due to IL-12. (*P < 0.05; **P < 0.01; ****P < 0.0001, ANOVA with Tukey multiple comparisons test).

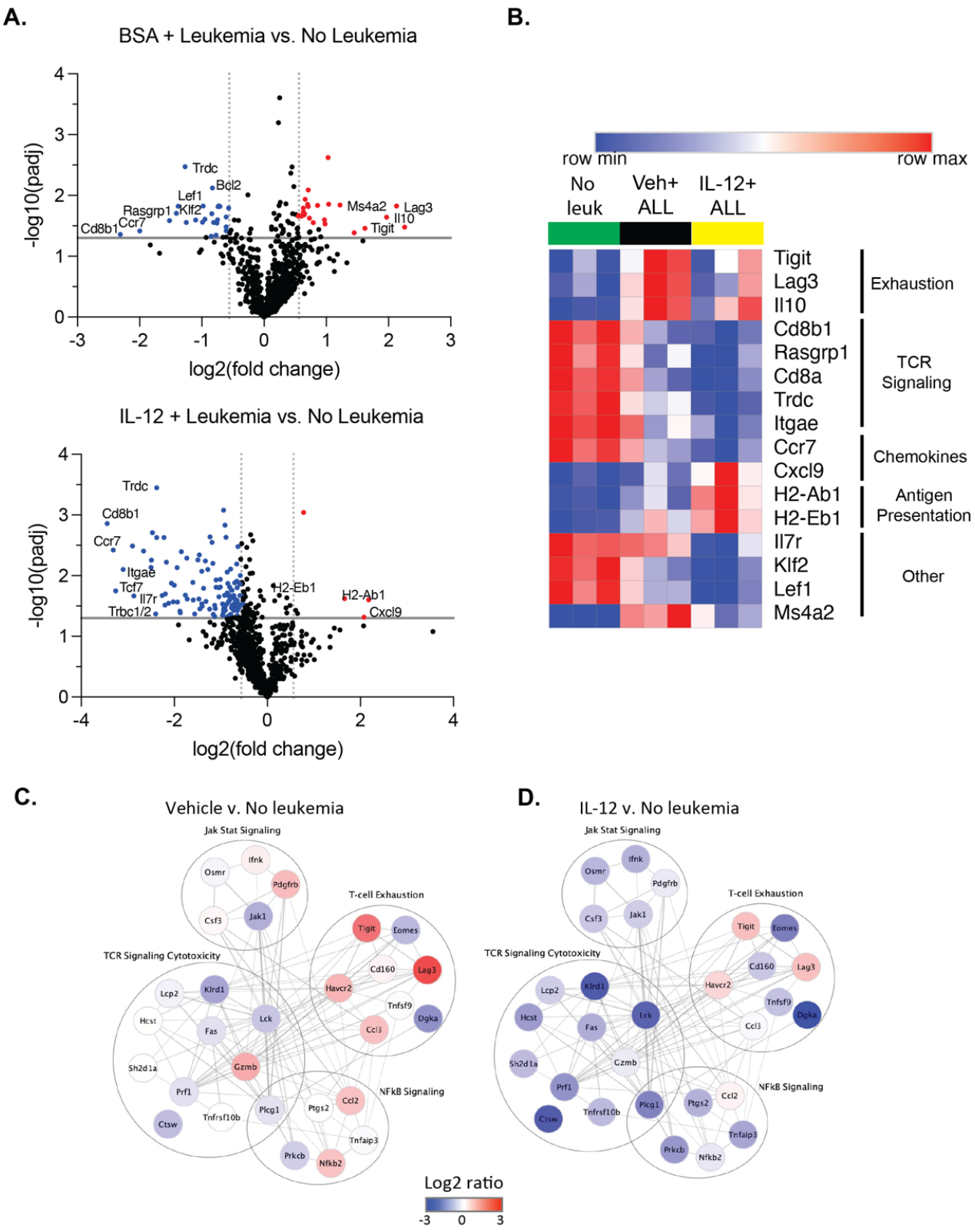
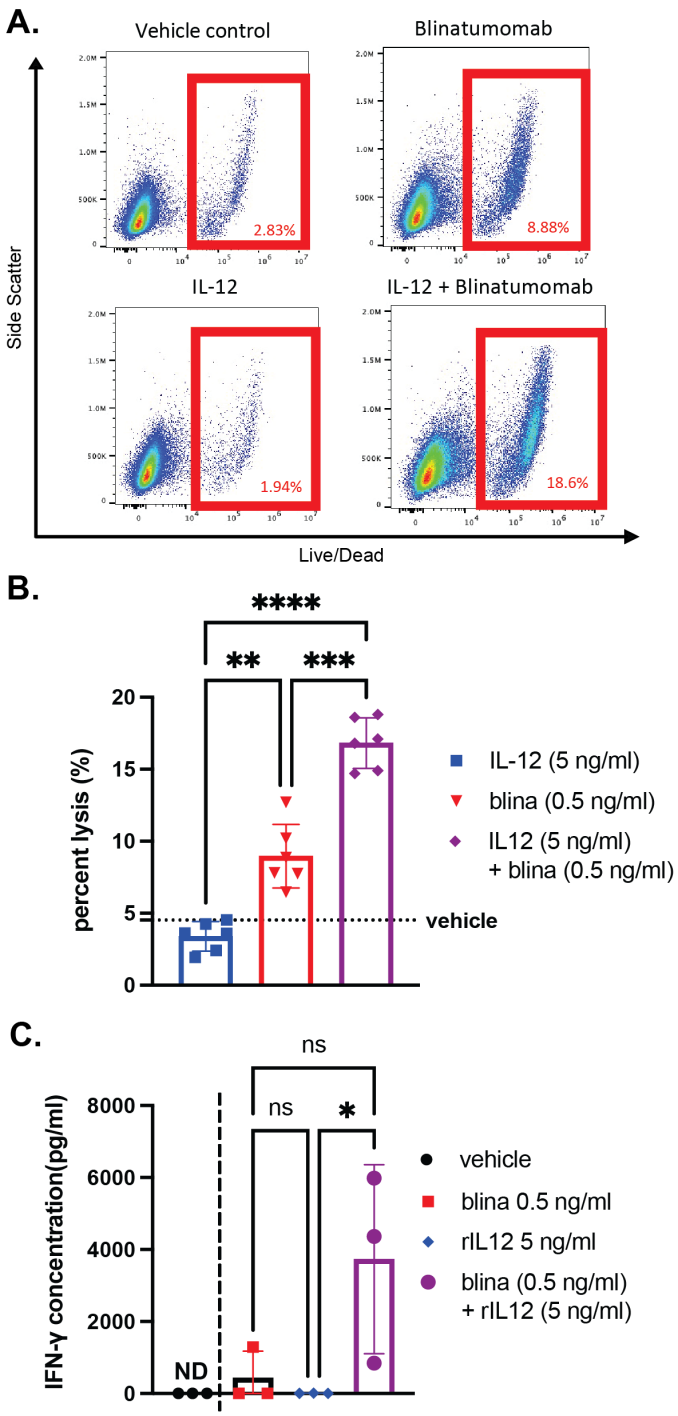

A.
BSA + Leukemia vs. No Leukemia
-log10(padj)
log2(fold change)
Trdc
Bcl2
Lef1
Rasgrp1
Klf2
Ccr7
Cd8b1
Ms4a2
Lag3
Il10
Tigit
IL-12 + Leukemia vs. No Leukemia
Trdc
Cd8b1
Ccr7
Itgae
Tcf7
Il7r
Trbc1/2
H2-Eb1
H2-Ab1
Cxcl9
B.
row min
row max
No leuk
Veh+ ALL
IL-12+ ALL
Tigit
Lag3
Il10
Cd8b1
Rasgrp1
Cd8a
Trdc
Itgae
Ccr7
Cxcl9
H2-Ab1
H2-Eb1
Il7r
Klf2
Lef1
Ms4a2
Exhaustion
TCR Signaling
Chemokines
Antigen Presentation
Other
C.
Vehicle v. No leukemia
Jak Stat Signaling
T-cell Exhaustion
TCR Signaling Cytotoxicity
NFkB Signaling
Log2 ratio
D.
IL-12 v. No leukemia
Jak Stat Signaling
T-cell Exhaustion
TCR Signaling Cytotoxicity
NFkB Signaling

Figure 4.5. Genes associated with T-cell exhaustion are induced in B-ALL-bearing mice.

Murine T-cells were isolated from the bone marrow of either untreated or rIL-12 treated WT mice at day 7 and subjected to targeted RNA sequencing using the nCounter Immune Exhaustion panel (Nanostring). **A.** Volcano plots of gene expression changes in T-cells from vehicle treated (top) or IL-12 treated (bottom) mice compared to mice without leukemia. **B.** Heat map demonstrating relative gene expression of indicated genes **C, D.** Protein-protein interaction networks with functional annotation from differentially expressed genes (FC >= 1.5, $p < 0.05$).

Figure 4.6. Blinatumomab and IL-12 combination therapy enhances T-cell cytolytic activity.

A. Representative flow cytometry plots of T-cell cytolytic activity exhibiting leukemia cell death based on treatment. **B.** Leukemia cell (Nalm6) death from T-cell cytolytic killing from 6 human donors (*, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$, ANOVA with Tukey multiple comparisons test). **C.** ELISA used to measure IFNγ secretion from human $CD3^+$ T-cells co cultured with Nalm6 cells (n=3 donors; *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$, ANOVA with Tukey multiple comparisons test).



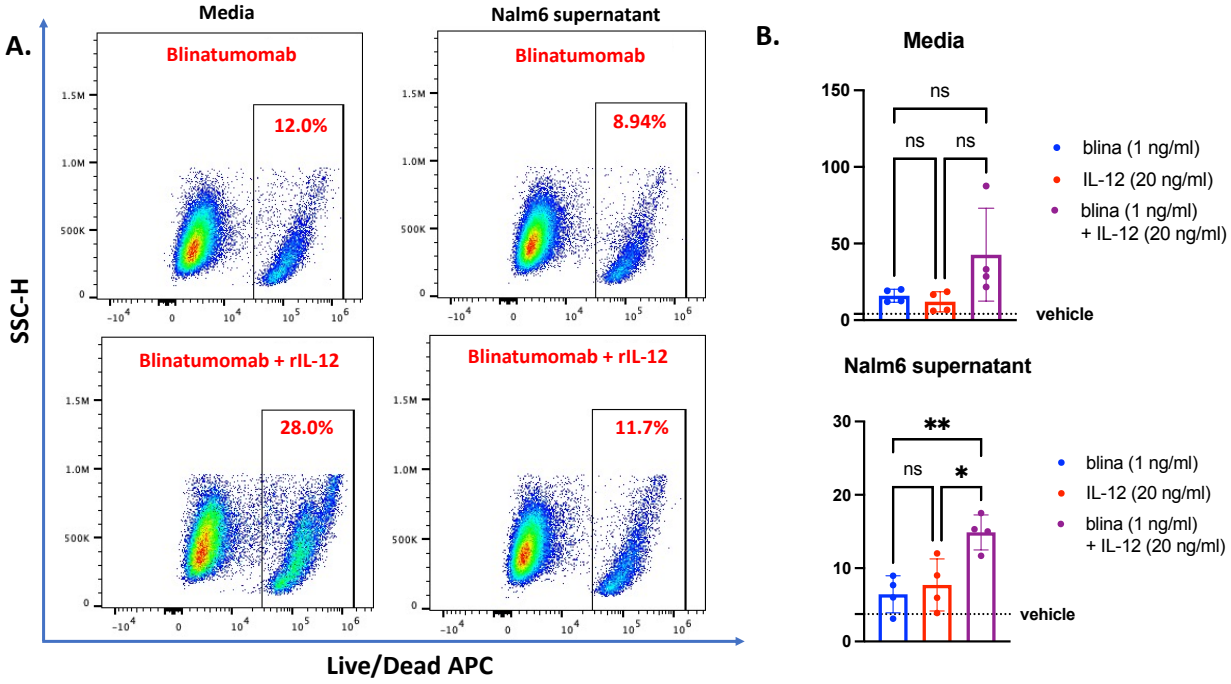


Figure 4.7. IL-12 overcomes reduced blinatumomab efficacy in the B-ALL secretome. **A.** Representative flow cytometry plots of T-cell cytolytic activity showing leukemia cell death in different experimental conditions. **B.** Leukemia cell death from T-cell cytolytic killing cultured in either control media (top panel) or Nalm6 supernatant (bottom panel) (n=4 donors; $*P < 0.05$; $**P < 0.01$; $****P < 0.0001$, ANOVA with Tukey multiple comparisons test).

Chapter 5: Conclusions

5.1 Summary and conclusions

Despite improved treatment strategies, leukemia remains a global health problem due to high relapse because of treatment failure rates. Even with immunotherapies there is still a need to elicit more effective long-term outcomes for patients. Also, the need to identify leukemic cell strategies to abrogate immune suppression in the bone marrow microenvironment is important for the field of personalized immunotherapy. As demonstrated, MRD positive patients have a more exhausted immune phenotype compared to MRD negative patients with B-ALL. In addition, in both human and murine models T-cell suppression occurs by B-ALL secreted factors. Thus, the overarching goal of this research work was to identify mechanisms of immune suppression and treatment strategies to overcome them in B-ALL.

Importantly, we demonstrated the potent activity of IL-12 to elicit an immunostimulatory microenvironment, abrogating immune suppression by B-ALL (**Fig. 5.1**). We identified that calcineurin depletion of leukemia cells resulted in an immunostimulatory microenvironment by the induction of cytokines, specifically IL-12, and genetic alterations that led to increased immunogenicity of B-ALL cells and a heightened immunostimulatory microenvironment. Further examination of the effects of IL-12 in our *in vivo* B-ALL model revealed that cytokines and chemokines significant for myeloid and T-cell activation, as well as trafficking, were upregulated in IL-12-treated mice compared to vehicle treated mice. Increased DC and $CD8^+$ T-cell numbers and subsequent activation of DCs exhibiting enhanced antigen priming due to IL-12 administration. Further, the novel combination therapeutic treatment in conjunction

with blinatumomab to overcome immune suppression demonstrates the potency and potential of IL-12.

Thus, this my work demonstrates the promise of IL-12 as an immunotherapeutic adjuvant for relapse or refractory ALL.

5.2 Future directions

We have made the novel discovery that the B-ALL secretome suppresses T-cell mediated immunity. To our knowledge, this is the first report of this phenomenon. Thus, we intend to identify specific secreted factors by B-ALL cells which suppress T-cell function using murine models and patient samples. Preliminary experiments to corroborate this data has been conducted to identify which type of secretory factor is responsible. I have found that B-ALL supernatant treated with either a lipase or nuclease reduces T-cell activation (**Fig. 5.2**); however, the specific factors are still unknown. Identifying secretory factors that can be targeted in B-ALL in combination with other immune therapeutic targets may improve treatment efficacy for patients who experience relapse or refractory disease.

Further, although the role of myeloid cells in B-ALL context has been described as immune-suppressive specifically, MDSCs and TAMs, the impact of immunogenic myeloid cells (M1) can potentially be leveraged to enhance therapeutics in B-ALL [97, 98, 101, 220]. This has mainly been studied in solid tumor models. Preliminary work demonstrated suppression of LPS-stimulated murine macrophage cell line, RAW 264.7, by the B-ALL secretome, represented by a decrease in markers of macrophage activation, CD80 and CD86 (**Fig 5.3)**. Currently leveraging myeloid cells to enhance immunogenicity in the B-ALL microenvironment has not been studied extensively, only the role of MDSCs [221]. Based on previous data showing myeloid cells are important for

leukemia control, M1 macrophages can be harnessed to elicit an immunogenic response in B-ALL. Examining the contribution of M1 macrophages on mutated B-cells will contribute to treatment strategies to consider for patients with B-ALL.

Regarding T-cells, previous studies in patients with B-ALL with relapse or refractory disease exhibited the Treg proportion may be a potential indicator of blinatumomab efficacy in these patients. In examination of peripheral blood, patients who responded to blinatumomab exhibited a reduced percentage of Tregs (4.82%) compared to non-responders (10.25%). However, *in vitro* depletion of Tregs resulted in enhanced activation of T-cells, exhibiting the immune suppressive nature of Tregs on the immune response to B-ALL [222]. We also demonstrated reduced efficacy of blinatumomab in the presence of B-ALL secretome, yet improved efficacy of this therapy in combination with IL-12 (**Fig. 4.7B**). This is a novel consideration for improving BiTEs therapeutic efficacy. A major downside to BiTE therapy is cytokine release syndrome in which a severe proinflammatory response is elicited [104]. Toxicity is also a consideration with IL-12 treatment [149, 182,183], and must be considered in the combinatorial therapy of IL-12 and blinatumomab. Therefore, there is a need to develop localized, less toxic methods of delivery for these immune therapeutics.

5.3 Clinical implications for localized delivery of IL-12 in B-ALL

Developing local, persistent IL-12 is the next step for clinical considerations of this cytokine as an immunotherapeutic. Local IL-12 delivery can significantly enhance IL-12 concentrations in the tumor microenvironment [223, 224]. Another benefit of localized delivery of IL-12 is the initiation of an immune response at the site of the tumor resulting in a systemic immune response. Systemic delivery can result in significant adverse events

by off-target interactions. Localized delivery of IL-12 has shown the generation of not only adaptive immunologic memory, but also prevention of relapse [225, 226].

Additionally, IL-12 has pleiotropic effects that result in unintended consequences. It induces the release of pro-inflammatory cytokines including IFN-γ, IL-6, and TNF-α [227]. Yet, if controlled, this can engage multiple immune response mechanisms, including activation of $CD8^+$ T -cells and NK cells and subsequent induction of production of IFN-γ resulting in tumor clearance. Also, a major hindrance to the effectiveness of cancer immunotherapies is the immune suppressive microenvironment. Local administration of IL-12 can reverse tumor-supporting immunosuppression resulting in strong antigenic-specific T-cell responses. High intra-tumoral concentrations of IL-12 have demonstrated a reversal of the immune suppressive phenotype of TAMs in the tumor microenvironment [214]. IL-12 has been shown to alter the phenotype of suppressive MDSCs as well [228].

Considering this evidence altogether, our group has worked in collaboration with Erik Dreaden, a principal investigator that conducts biomedical engineering research. The Dreaden lab has developed a rapid assembly and screening method of multivalent immune cell redirection (ICR) drug candidates to redirect the lytic activity of T-cells toward leukemic B-cells while simultaneously co-delivering T-cell-stimulating IL-12, termed bispecific T-cell engaging cytokines (BiTEokines) (Fig. 5.4) [219]. This is a localized delivery method to target leukemia cells. Based on previous studies, several BiTEokine candidates were identified and tested *ex vivo*. Two lead compounds, 35 and 37, exhibited a >17-fold increase in lytic activity [219]. Further work to examine efficacy of this localized nanoparticle therapeutic *in vivo* are currently underway. This may have

significant clinical impact to deliver IL-12 locally in combination with a current clinical therapeutic, blinatumomab, to prevent relapse in patients with B-ALL.

In conclusion, work presented in this thesis demonstrates that IL-12 plays a significant role leukemia immunosurveillance. Furthermore, I demonstrate that this cytokine augments the efficacy of B-ALL targeting immunotherapies by overcoming the immunosuppressive B-ALL secretome. Developing methods to deliver IL-12 locally in combination with other therapies has great promise. Ongoing clinical trials are attempting to determine the efficacy of localized IL-12 delivery strategies [224 – 226]. Our own work has demonstrated the efficacy of localized combination immunotherapies for IL-12 [219]. These studies will significantly contribute to the new era of precision medicine which will result in more effective and less toxic treatment strategies for patients with cancer. Ultimately, I hope this work is a major step toward improving the quality of life for cancer patients.

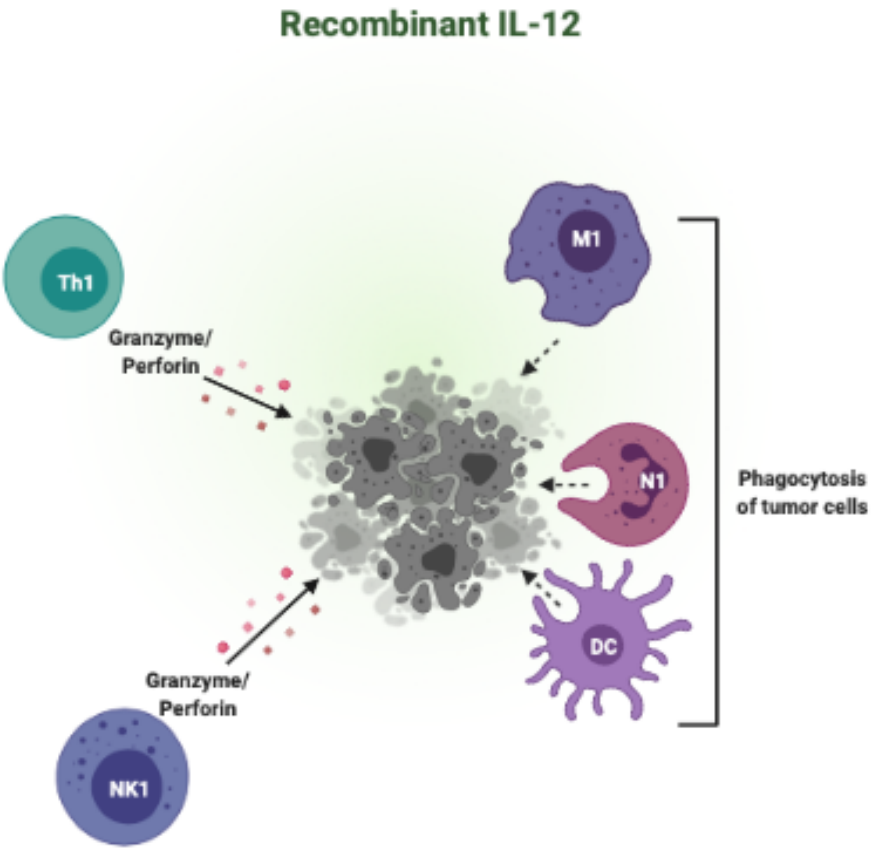


Figure 5.1. Recombinant IL-12 stimulates immune response leading to leukemic cell death. Adapted from Biorender.

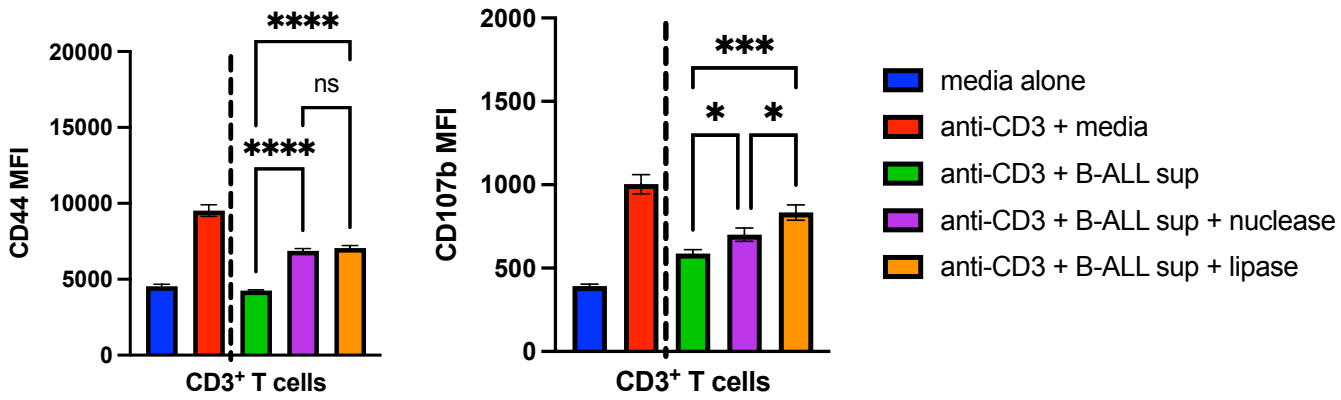


Figure 5.2. Lipid or nucleic acids may be responsible for T cell suppression in B-ALL secretome. Stimulated T-cells were cultured in either media or B-ALL supernatant and treated with either a nuclease or lipase.

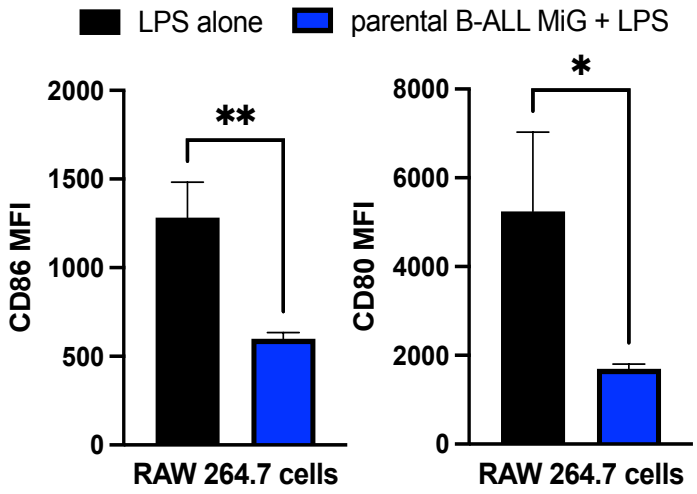


Figure 5.3. B-ALL secretome suppresses macrophage activation. RAW 264.7, a murine macrophage cell line, were stimulated with LPS and cultured in the presence of B-ALL supernant or media alone for 48 hrs.

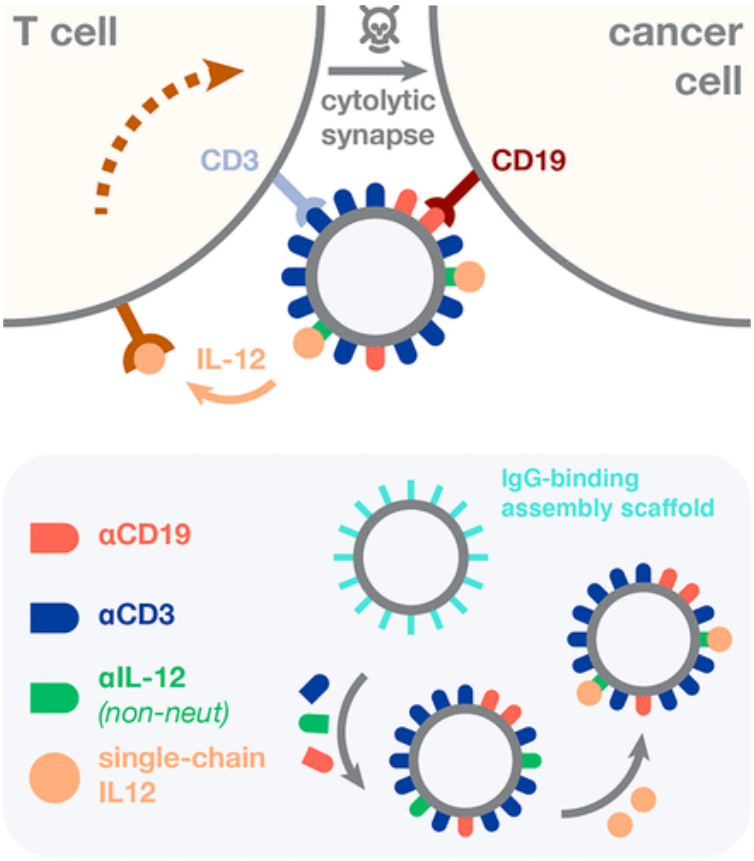


Figure 5.4. Structure and assembly of bispecific T cell engaging cytokines (BiTEokines). Adapted from Do, P. et al. Rapid Assembly and Screening of Multivalent Immune Cell-Redirecting Therapies for Leukemia. *ACS Comb Sci.* **22**, 533-541 (2020). Schematic of drug-induced synapse formation between T-cells and leukemic B cells, as well as synapse-targeted delivery of the cytokine, IL-12. Inset illustrates the modular and rapid self-assembly of CD19 × CD3 × IL12 BiTEokines via addition of human IgG to protein G-conjugated iron oxide nanoparticles and subsequent cytokine complexation. The solid beige arrow denotes cytokine release or trans-presentation.

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